REMARKS

Claims 78-86 and 93-101 are pending. Claims 93, 99, and 100 have been amended. Claims 102 and 103 have been added. Claims 78-86 and 93-103 remain in the case.

Interview and QAS meeting summary

Applicant would like to thank Examiner Crowder for the telephonic interview of August 30, 2007, and for the report by Examiners Haddad and Crowder that a interiview has been scheduled with the QAS on the written description issue of record. Applicant also would like Examiners Gambel, Crowder, Haddad and Brumback for taking the time to meet personally on December 3, 2007 to discuss this case. In advance of this meeting, applicant made of record the declarations under Rule 1.132 of two experts.

At a previous interview on May 17, 2007 with Examiners Gambel and Crowder, the written description issue also was discussed. Applicant understood the result of that interview to be that the rejection for lack of written description would be obviated by amending the claims to recite "B-cell antibodies" instead of "antibody or antibody fragment specific to a marker associated with a B cell." Indeed, the Interview Summary for that meeting states that "Applicant will consider filing a RCE and to amend the claims to recite antibodies to B cells rather than 'a marker associated with a B cell' *to obviate WD rejection of record*" (emphasis added). However, in the next action, claims amended in this fashion were again rejected under Section 112 for lack of written description.

At the meeting with the QAS, Examiner Brumback felt that applicant's case would be strengthened greatly if applicant could identify B-cell antibodies available at the time of applicant's invention in 1992 which subsequently had been demonstrated to be effective in the treatment of autoimmune diseases. The undersigned would like to thank Examiner Brumback for this constructive suggestion, and applicant will provide evidence of this in the present response, and will discuss in detail the import of the Rule 1.132 declarations that have been made of record in this case.

Rejection of claims 78-86 and 93-101 under the second paragraph of Section 112 based on the term "immune disease"

Claims 78-86 and 93-101 are rejected under the second paragraph of Section 112 based on the recitation of "an immune disease." The examiner urges that the metes and bounds of this

term is unclear and ambiguous. In this regard she argues that the term "can encompass disease with immune system being positively (*e.g.*, autoimmune) or negatively (*e.g.*, HIV) regulated.

In order to address this position on the part of the examiner, applicant sought the input of an expert in the field, and more particularly the input of an expert in the field who was familiar with the terminology circa 1992, the relevant time frame to be considered with respect to the present case. Applicant has provided the declaration of Dr. Dörner, an expert in the field of autoimmune diseases.

Dr. Dörner takes exception to the examiner's statement that the term "immune disease" would encompass both positive and negative regulation of the immune system. He notes that the terms "positively" versus "negatively" regulated have never been widely accepted by the scientific community, and current terminology, introduced in the early 2000s, refers to "disturbances in homeostasis of the immune system." As to diseases such as HIV, he explains that there are a lot of otherwise defined diseases such as infections (HIV, other viruses, bacteria), malignant diseases (lymphoma), etc., which impair or affect the immune system, but that immune activation by all of these has a defined cause. By contrast, he notes that the classical term "immune disease," circa 1992, relates to **idiopathic** disorders of the immune system. These are the so-called classical autoimmune diseases for which the cause of immune activation was unknown.

Dr. Dörner is extremely well qualified to opine on the issue of the scope of the term immune disease circa 1992, and he attests, as an immunologist and rheumatologist, that in the context of the entire disclosure of the present application, he finds the term immune disease to be neither unclear nor ambiguous. He emphasizes that he certainly would *not* understand the term to include disease in which the immune system is negatively regulated, such as HIV. In this regard, he notes that the term is used in conjunction with a discussion of the use of a B-cell antibody and also in conjunction with a disclosure of the ablation of normal spleen cells and a disclosure of "certain immune diseases, such as immune thrombocytopenic purpura," and he immediately recognized that the reference in the disclosure of "antibodies that target the spleen," is a reference to a targeting of immune cells that reside in the spleen. He explains that B-cell hematologic abnormalities are a consequence of immune diseases in which the immune system is positively regulated, and immune thrombocytopenic purpura (ITP) is an example of such an immune disease. In particular, he points out that B cells differentiating into plasma cells are known to make antibodies, including the autoantibodies considered to be responsible for destroying platelets in ITP. Therefore, he says he had no difficulty in ascertaining the scope of

the term "immune disease" in the context of the present disclosure as referring to classical autoimmune diseases, and would not understand the term to include diseases such as HIV in which the immune system is "negatively regulated"[1] and had no difficulty in determining the scope of the present claims.

Based on the foregoing highly probative evidence, it is clear that the term "immune disease" is not unclear or ambiguous. During the meeting on December 3rd, Examiner Gambel noted that other applicants sometimes define "immune disease" as broadly as noted by Examiner Crowder in her Action. As set forth in MPEP 2111.01:

> It is well established that an applicant is entitled to be his or her own lexicographer and may rebut the presumption that claim terms are to be given their ordinary and customary meaning by clearly setting forth a definition of the term that is different from its ordinary and customary meaning(s). See *In re Paulsen*, 30 F.3d 1475, 1480, 31 USPQ2d 1671, 1674 (Fed. Cir. 1994) (inventor may define specific terms used to describe invention, but must do so "with reasonable clarity, deliberateness, and precision" and, if done, must "'set out his uncommon definition in some manner within the patent disclosure' so as to give one of ordinary skill in the art notice of the change" in meaning) (quoting *Intellicall, Inc. v. Phonometrics, Inc.*, 952 F.2d 1384, 1387-88, 21 USPQ2d 1383, 1386 (Fed. Cir. 1992)). Where an explicit definition is provided by the applicant for a term, that definition will control interpretation of the term as it is used in the claim. *Toro Co. v. White Consolidated Industries Inc.*, 199 F.3d 1295, 1301, 53 USPQ2d 1065, 1069 (Fed. Cir. 1999) (meaning of words used in a claim is not construed in a "lexicographic vacuum, but in the context of the specification and drawings").

However, the fact that "others" may have defined a term in a way that is inconsistent with the plain meaning to be given a term is not relevant, and is a red herring. Where an applicant does not set forth a definition of a term that "is different from its ordinary and customary meaning" then the "ordinary and customary meaning" must be used. MPEP 2111.01 goes on to note that (emphasis has been added):

> [T]he ordinary and customary meaning of a claim term is the meaning that ***the term would have to a person of ordinary skill in the art in question at the time of the invention, i.e., as of the effective filing date of the patent application.***" *Phillips v. AWH Corp.*, *>415 F.3d 1303, 1313<, 75 USPQ2d 1321>, 1326< (Fed. Cir. 2005) (*en banc*). *Sunrace Roots Enter. Co. v. SRAM Corp.*, 336 F.3d 1298, 1302, 67 USPQ2d 1438, 1441 (Fed. Cir.

[1] Dr. Dörner notes that he here adopts "the little-used and imprecise terminology employed in the Office Action."

> 2003); *Brookhill-Wilk 1, LLC v. Intuitive Surgical, Inc.*, 334 F.3d 1294, 1298 67 USPQ2d 1132, 1136 (Fed. Cir. 2003)("In the absence of an express intent to impart a novel meaning to the claim terms, the words are presumed to take on the ordinary and customary meanings attributed to them by those of ordinary skill in the art."). ***It is the use of the words in the context of the written description and customarily by those skilled in the relevant art that accurately reflects both the "ordinary" and the "customary" meaning of the terms in the claims.*** *Ferguson Beauregard/Logic Controls v. Mega Systems*, 350 F.3d 1327, 1338, 69 USPQ2d 1001, 1009 (Fed. Cir. 2003)

A number of points should be apparent from the foregoing:

1. When a patentee acts as his own lexicographer in redefining the meaning of particular claim terms away from their ordinary meaning, he must clearly express that intent in the written description.

2. In the absence of a clearly expressed intent to use a definition that is contrary to the ordinary meaning, terms are to be given their plain meaning.

3. The appropriate time frame for determining the plain meaning of a term is the filing date of the invention.

4. The appropriate person to determine the plain meaning of a term is the person who was skilled in the art at the time of the filing date.

5. The specification should also be relied on for more than just explicit lexicography or clear disavowal of claim scope to determine the meaning of a claim term when applicant acts as his or her own lexicographer; and the meaning of a particular claim term may be defined by implication, according to the usage of the term in the context in the specification.

In the present case, applicant has indicated no unusual definition for the term "immune disease" and therefore the appropriate meaning is the plain meaning in use in 1992. The most appropriate person for determining the plain meaning in 1992 is a person of skill in the art, particularly one who was skilled in the art in that time frame. In arriving at the determination of the plain meaning to be accorded a term in a claim, the usage of the term in the context in the specification is to be considered. Dr. Dörner is a person skilled in the art, and he was active in the filed of immune diseases in the relevant time frame. He notes both the plain meaning of the term "immune disease" at the time, and bolsters his conclusion with reference to the context in which the term is used in the specification. Therefore, the evidence in the form of Dr. Dörner's declaration is the most probative evidence available on the point raised by the examiner.

Moreover, Dr. Dörner's conclusion is echoed by that of Dr. Kenneth Foon, who also has provided a declaration under Rule 1.132. Dr. Foon finds the term "immune disease" in the present application and the claims to mean autoimmune diseases. Thus, the term "immune disease" is not unclear or unambiguous, and reconsideration and withdrawal of this rejection under the second paragraph of Section 112 is respectfully requested.

As suggested by the examiner, applicant proposes claim 102, which recites that the immune disease is a B-cell immune disease, as supported by the specification at page 12, lines 30-35.

Rejection of claim 93 under the second paragraph of Section 112 based on the recitation of "an antibody having multiple epitope"

Claim 93 has been amended to recite an antibody which binds multiple epitopes or antigens, which has been indicated by the examiner as resolving this rejection. Basis for the amendment is found on page 14, lines 7-15.

Rejection of claims 78-86 and 93-101 the first paragraph of Section 112 for lack of written description

Claims 78-86 and 93-101 are rejected under the first paragraph of Section 112 for lack of written description, the examiner alleging that the instant specification does not describe the subject matter of the claims in such a way that it reasonably conveys to a person of skill in the art that applicant had possession of the claimed invention at the time of filing. As with the rejection under the second paragraph of Section 112, discussed above, the appropriate person to inform the decision of whether applicant was in possession of his claimed invention at the time of filing is a person of skill in the art, particularly one who was skilled in the art at the time of filing of the application. In order to provide evidence on this point, applicant has submitted a declaration under Rule 1.132 by Dr. Kenneth Foon. Dr. Foon is an expert in the field of B-cell antibodies. In fact, during the meeting on December 3rd, Examiner Gambel noted that he had often, in the course of examining various cases, cited Dr. Foon's seminal review on B-cell antibodies which is referenced in Dr. Foon's declaration.

Dr. Foon begins by noting the examiner's concerns with issues of whether the disclosure must include evidence of "relevant identifying characteristics" and/or "a disclosed correlation between function and structure" for B-cell antibodies in order to sufficiently describe the invention to a skilled artisan. He also identifies her concern that the method depends upon "finding 'B-cell antibody'" and that "without such an antibody, the skilled artisan cannot practice the claims method of treating an immune disease." Finally, he notes that the examiner says that the claims

call for B-cell antibodies or fragments generally and, in her words, "[lack] a common structure essential for the function (*e.g.* antigen specificity) and the claims do not require any particular structure basis or testable function be share by the instant 'B-cell antibody or fragment thereof.'" Dr. Foon then sets forth, in great detail, the reasons why he "does not believe these concerns to be well-founded scientifically."

Dr. Foon begins with a primer on the CD classification system, according to which a proposed surface molecule is only assigned a CD number once two specific monoclonal antibodies have been shown to bind to the molecule. He references both the 4th and the 5th International Workshops. While only the former was published before the filing date of the present application, it shows numerous B cell CDs, each of which must, by convention, correspond to at least two different B-cell antibodies that identify the particular CD.

In addition, Dr. Foon identifies reports of several other B-cell antibodies that had been disclosed prior to the 1992 filing date of the present application. These include an anti-B1 antibody reported by both Stashenko and Nadler, and a chimeric anti-CD20 antibody, 2H7, reported by Liu. Dr. Foon also highlights the anti-CD20 antibody 1F5 that was used by Press to treat malignant lymphoma and the NHL. Press also used the anti-CD37 antibody MB1 to treat patients with NHL. Dr. Foon also cites his 1987 article, which discloses that B-cell antibodies are useful in monoclonal antibody therapy of B-cell cancers, citing one study in which patients were treated with the BA-1, BA-2 and BA-3 monoclonal antibodies to B cells, and another in which patients were treated with anti-B1antibody. Based on these studies, Dr. Foon concludes that B-cell antibodies have been demonstrated to possess a commonality of function both in terms of their ability to specifically bind to B cells and also in the ability to affect disease progression as a result of that binding.

Dr. Foon notes that the anti-CD20 antibody 2H7 was patented by Robinson *et al.*, based on an application filed in 1987, and that Ledbetter *et al.* filed an application in 1986 that disclosed the antibody G28-5 which defined the B-cell receptor Bp50. Each of these applicants deposited a hybridoma which secreted their claimed antibody.

Based on the foregoing, Dr. Foon attests that he would understand that the applicant in the present case:

> was in possession of a method of using B-cell antibodies generally to treat immune diseases, and not just the LL2 B-cell antibody specifically. The skilled artisan would understand that the contribution to the art was the teaching that B-cell antibodies generally could be used to treat immune diseases. These B-cell

> antibodies have a commonality of function, in that they all bind to B-cell surface antigens. In another context, that of B-cell cancers, this commonality of function has been found to correlate to an ability to affect disease progression as a result of that binding (I have discussed this in paragraph 7 above). This binding function is one that is testable, as I described in paragraph 3 above, and the skilled artisan would not need to know the structure of particular B-cell antibodies in order to be apprised of, and to practice, the full scope of this invention.

This conclusion is repeated in the concluding paragraph of Dr. Dörner's declaration.

Accordingly, the present specification provides a written description of the invention recited in the present claims, *i.e.*, the treatment of immune diseases with B-cell antibodies, and conveys to one skilled in the art that applicant had possession of that invention at the time of filing.

Evidence that B-cell antibodies which existed in 1992 are effective in treating autoimmune disease

As noted at the outset, Examiner Brumback felt that evidence that B-cell antibodies in existence at the time of filing of the present application had subsequently been shown to be effective in the treatment of an autoimmune disease would be highly informative with respect to the Section 112 issue of record. Applicant therefore has researched the literature in order to place such evidence into the record.

Several of the B-cell antibodies known in 1992 have been tested for efficacy in treating autoimmune diseases. These include epratuzumab. Epratuzumab is a humanized version of the anti-CD22 antibody LL2 which is mentioned in applicant's specification. Dörner *et al.*, *Arthritis Res Ther.* 2006; 8(3): R74 reports an initial clinical trial of epratuzumab (humanized anti-CD22 antibody) for immunotherapy of systemic lupus erythematosus. Steinfeld *et al.* report that "recent studies have demonstrated the efficacy and safety of epratuzumab in several autoimmune diseases, including systemic lupus erythematosus and primary Sjögren's syndrome." *Expert Opin Biol Ther.* 2006 Sep;6(9):943-9. Copies of both of these articles are appended.

Trubion has a Fab-based product made from 1F5, another B-cell antibody that was known in 1992, as evidenced by Dr. Foon's declaration. This antibody is being tested in rheumatoid arthritis by Wyeth, who licensed the antibody. A press release from Trubion is appended.

A humanized version of the anti-CD20 antibody 2H7 also has been shown to be effective in treating autoimmune diseases. 2H7 is another antibody referenced in Dr. Foon's declaration. This antibody, named ocrelizumab, is being tested in the treatment of rheumatoid arthritis. Genentech has announced that ocrelizumab met its primary endpoint of safety in all doses studied and also met its secondary endpoint of clinical activity at all dose levels studied in a Phase I/II ACTION study of rheumatoid arthritis.

Thus humanized versions of three antibodies known in 1992 have been shown to be effective in the treatment of various autoimmune diseases. This further supports the patentability of the present claims which are directed to the treatment of immune diseases with B-cell antibodies.

Rejections of claims under Section 102

Claims 78, 81-86 and 97-100 are rejected under Section 102(b) based on Meyer *et al.* (US 4,861,579).

Meyer's only specific antibodies are Lym-1 and Lym-2. Although Meyer's results with Lym-1 and Lym-2 show some B-cell activity, these are HLA-DR antibodies. The literature clearly shows that this class of antibodies reacts with more than just B-cells, and even with some solid tumors. For example, Würflein *et al.* 1998 evaluated antibodies for their capacity to induce cell-mediate lysis of malignant B cells, and "compared killing mediated by chimeric IgG 1 antibodies with that from FcγRI-directed bispecific antibodies, targeting classical HLA class II, or the Lym-1 and Lym-2 antigens. The latter two are variant forms of HLA class II, which are highly expressed on the surface of malignant B cells but which are found only at low levels in normal cells."

Würflein *et al.* further notes that "Lym-I and Lym-2 may have the additional advantage that they bind preferentially to HLA class II in malignant human B cells compared to normal B cells and monocytes." Epstein *et al.* 1987, which is referenced in Meyer *et al.*, cites facts in agreement with the low level of expression on normal cells and with the HLA-DR specificity of these antibodies noted by Würflein *et al.* Low levels of binding to normal B cells leads to the conclusion that the antibodies would *not* be effective for treating immune diseases.

The only species of antibodies disclosed by Meyer *et al.* are not B-cell antibodies (Meyer's characterization being inaccurate in this regard) and they are expressed only at very low levels on normal cells. This is in clear contrast to applicant's disclosed species of EPB2, which is a B-cell antibody and is well expressed on normal B cells. Unlike applicant's disclosure, Meyer's disclosure does not provide a written description that evidences possession of an

invention of treating autoimmune diseases with B-cell antibodies. Copies of Würflein *et al.* and Epstein *et al.* are appended.

Claims 78, 79, 81, 93, and 96 are rejected under Section 102(b) based on Bussel *et al.* as evidenced by Grandamont *et al.* Bussel *et al.* is cited as showing the treatment of ITP with IVIG. Grandamont *et al.* is cited as evidencing that IVIG includes antibodies reactive against antigens on B lymphocytes. In this regard, the examiner cites "the entire document" but particularly page 3065. Upon careful review of Bussel *et al.*, applicant is unable to find any disclosure that IVIG includes antibodies against B lymphocytes. The role of the Fc region of IVIG is mentioned on page 3065, and then the potential contribution of anti-idiotype antibodies, but there is no disclosure that IVIG includes B-cell antibodies. Moreover, the model used in Grandamont was "an in vitro culture system that reproduces T-dependent activation of B lymphocytes through the binding of CD40 on B lymphocytes." CD40 is a receptor molecule on the cell surface of many cell types, including monocytes, dendritic cells (in the nervous system), endothelial cells (within blood vessels), and epithelial cells, in addition to mature B cells and most B-cell malignancies. Thus, Grandamont *et al.* fails to provide the evidence urged that IVIG contains B-cell antibodies, and further suggests that the effects observed for IVIG have to do with T-dependent activation of B lymphocytes by an unidentified mechanism. Therefore, the rejection based on Bussel *et al.* as evidenced by Grandamont *et al.* is *prima facie* defective and reconsideration and withdrawal is respectfully requested.

Rejections of claims under Section 103

Claims 78, 80, 82-86, 95 and 101 are rejected under Section 103 based on Meyer *et al.* in view of Sivam *et al.* (US 5,116,944). Meyer *et al.* is discussed above. Sivam *et al.* is cited as teaching antibody fragments and antibody conjugated to a cytokine. Sivam *et al.* fails to overcome Meyer's failure to teach treatment of an immune disease with a B-cell antibody. No *prima facie* case of obviousness exists.

Claims 78 and 94 are rejected under Section 103 based on Meyer *et al.* in view of Fishwild *et al.* 1996. Fishwild *et al.* is cited as teaching human monoclonal antibodies. Fishwild *et al.* fails to overcome Meyer's failure to teach treatment of an immune disease with a B-cell antibody. No *prima facie* case of obviousness exists.

In view of the foregoing, it is believed none of the references, taken singly or in combination, disclose the claimed invention, and therefore a notice of allowance is respectfully requested. If there are any problems with this response, or if the examiner believes that a

telephone interview would advance the prosecution of the present application, Applicant's attorney would appreciate a telephone call.

Respectfully submitted,

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DECEMBER 26, 2007
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Trubion Pharmaceuticals Announces Presentation of Positive Data From Phase IIb and Re-treatment Studies With TRU-015 in Patients With Rheumatoid Arthritis

SEATTLE, Nov 08, 2007 /PRNewswire-FirstCall via COMTEX News Network/ -- Trubion Pharmaceuticals Inc. (Nasdaq: TRBN) today announced presentation of positive data from a Phase IIb study that showed that Trubion's TRU-015 for rheumatoid arthritis (RA) provided statistically significant efficacy after a single infusion of 800 mg or 1,600 mg. In addition, Trubion also announced presentation of data showing that repeat administration with TRU-015 was well tolerated and resulted in a consistent pharmacokinetic (PK) and pharmacodynamic (PD) profile. Trubion is co-developing TRU-015 with Wyeth Pharmaceuticals, a division of Wyeth (NYSE: WYE), for the treatment of rheumatoid arthritis.

These data will be presented during two poster sessions at the annual meeting of the American College of Rheumatology (ACR) on Nov. 8 and 9, 2007. The posters are available in the Events section of Trubion's Web site at http://investors.trubion.com/events.cfm.

"TRU-015's ability to significantly improve RA signs and symptoms following a single infusion could represent a new level of convenience for patients and physicians. These results also suggest that clinical responses may be maintained during B-cell recovery," said Peter Thompson, M.D., FACP, president, chief executive officer and chairman of Trubion. "We and our partner have agreed on a design for our next study that we believe could be supportive of a registration package, and we look forward to TRU-015's continued evaluation."

TRU-015 Improves RA Disease Activity in Phase II Trial (ACR Presentation L7)

On Sept. 10, 2007, Trubion announced preliminary analysis of results for its TRU-015 Phase IIb randomized, double-blind, placebo-controlled, multicenter clinical trial that included 276 patients with rheumatoid arthritis. Patients were randomized equally into five groups that received either placebo, 200 mg, 400 mg, 800 mg or 1,600 mg of TRU-015. The study was designed to evaluate the safety and efficacy of a single intravenous infusion of TRU-015 compared to placebo for a 24-week period.

Data announced previously showed the improvement in DAS-28 compared to placebo was statistically significant in the 800 mg dose group at 12 weeks and at all subsequent assessments, and in the 1,600 mg dose group at 16 weeks and at all subsequent assessments. At 24 weeks, ACR 20, 50 and 70 response rates in the 800 mg dose group were 65 percent, 26 percent and 0 percent, respectively. ACR 20, 50 and 70 response rates in the 1,600 mg dose

group were 61 percent, 13 percent and 4 percent, respectively. ACR 20, 50 and 70 response rates at 24 weeks in the placebo group were 33 percent, 9 percent and 2 percent, respectively.

At 24 weeks, significant improvement in the Health Assessment Questionnaire Disability Index (HAQ DI) was observed in the TRU-015 1,600 mg group (-0.70 v -0.37 [p=0.008]) and the 800 mg group (-0.64 v-0.37 [p=0.035]).

The HAQ DI measures patients' physical function in defined activities. Median C-Reactive Protein (CRP) improvement was 57 percent in the 1,600 mg group, 48 percent in the 800 mg group and 28 percent in the placebo group. The CRP test measures the concentration of a protein that is present during inflammatory episodes.

TRU-015 administered as a single dose was generally well tolerated, and only one subject in the 400 mg group experienced a grade 3 adverse event on the day of infusion.

Comparable Data Following Repeat Administration (ACR Presentation 309)

The objective of the re-treatment study was to evaluate the safety, PD, PK and immunogenicity of TRU-015 for RA with repeated doses after receiving initial administration in a Phase I/IIa study. Patients treated with a single course of 5 mg/kg or higher in a previously conducted TRU-015 Phase I/IIa study were eligible for re-treatment. Patients who received a single infusion of 5 mg/kg received a single infusion of 5 mg/kg upon re-treatment, and those who received higher doses of TRU-015 received a single infusion of 15 mg/kg upon re-treatment. PD response of B-cells was also evaluated after initial treatment and after re-treatment.

Fifty-four patients were eligible for re-treatment, and at the time of this analysis, re-treatment data were available for 36 patients. B-cell depletion and recovery following re-treatment with TRU-015 was comparable to that seen after initial treatment. Ongoing patient evaluations showed maintenance of ACR responses with repeated single doses of TRU-015 at six-month intervals through at least two retreatment courses. Total serum IgG levels remained within normal limits. In addition, subjects treated with three or more courses of therapy experienced persistent decreases in rheumatoid factor and IgM levels without experiencing decreases in IgG or IgA levels. No neutralizing antibodies to TRU-015 had been detected at the time of this assessment.

Re-treatment with TRU-015 was generally well tolerated, and no grade 3 or 4 adverse events occurred on the day of infusion.

Research article

Open Access

Initial clinical trial of epratuzumab (humanized anti-CD22 antibody) for immunotherapy of systemic lupus erythematosus

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Abstract

B cells play an important role in the pathogenesis of systemic lupus erythematosus (SLE), so the safety and activity of anti-B cell immunotherapy with the humanized anti-CD22 antibody epratuzumab was evaluated in SLE patients. An open-label, single-center study of 14 patients with moderately active SLE (total British Isles Lupus Assessment Group (BILAG) score 6 to 12) was conducted. Patients received 360 mg/m^2 epratuzumab intravenously every 2 weeks for 4 doses with analgesic/antihistamine premedication (but no steroids) prior to each dose. Evaluations at 6, 10, 18 and 32 weeks (6 months post-treatment) follow-up included safety, SLE activity (BILAG score), blood levels of epratuzumab, B and T cells, immunoglobulins, and human anti-epratuzumab antibody (HAHA) titers. Total BILAG scores decreased by ≥ 50% in all 14 patients at some point during the study (including 77% with a ≥ 50% decrease at 6 weeks), with 92% having decreases of various amounts continuing to at least 18 weeks (where 38% showed a ≥ 50% decrease). Almost all patients (93%) experienced improvements in at least one BILAG B- or C-level disease activity at 6, 10 and 18 weeks. Additionally, 3 patients with multiple BILAG B involvement at baseline had completely resolved all B-level disease activities by 18 weeks. Epratuzumab was well tolerated, with a median infusion time of 32 minutes. Drug serum levels were measurable for at least 4 weeks post-treatment and detectable in most samples at 18 weeks. B cell levels decreased by an average of 35% at 18 weeks and remained depressed at 6 months post-treatment. Changes in routine safety laboratory tests were infrequent and without any consistent pattern, and there was no evidence of immunogenicity or significant changes in T cells, immunoglobulins, or autoantibody levels. In patients with mild to moderate active lupus, 360 mg/m^2 epratuzumab was well tolerated, with evidence of clinical improvement after the first infusion and durable clinical benefit across most body systems. As such, multicenter controlled studies are being conducted in broader patient populations.

Introduction

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease that can involve many organ systems [1]. In Europe and the United States, estimates of the number of affected individuals range from 24 to 65 cases per 100,000 people [1,2]. The clinical course of SLE is episodic, with recurring activity flares causing increasing disability and organ damage. Cyclophosphamide, azathoprine, and corticosteroids remain important for long-term management of most patients having active disease, and even those in clinical remission [1]. Despite the important advances made with these drugs, especially cyclophosphamide, in controlling lupus disease activity, they have considerable cytotoxicity and cause, for example, bone marrow depression, ovarian failure, enhanced risk of bladder cancer, as well as the known side effects of long-term systemic corticosteroid therapy. As such, there continues to be a need for the development of targeted and less toxic therapies.

BCR = B cell antigen receptor; BILAG = British Isles Lupus Assessment Group; HACA = human anti-chimeric antibody; HAHA = human anti-human (epratuzumab) antibody; NCI CTC = National Cancer Institute Common Toxicity Criteria; NHL = non-Hodgkin lymphoma; SLE = systemic lupus erythematosus.

Specific autoantibodies against nuclear, cytoplasmic, and membrane antigens remain the serological hallmark of SLE. While lymphopenia is common, there is an increase in the level of activated B cells [3,4] and characteristic alterations of B cell subpopulations [5,6] that may be driven by extrinsic or intrinsic factors. B cells appear to have a key role in the activation of the immune system, in particular through the production of cytokines and by serving as antigen-presenting cells (reviewed recently in [7]). Although B cell activation can occur independently of T cell help in lupus, a substantial fraction of B cells is activated in a T cell dependent manner [8-10], as demonstrated by isotype switching and affinity maturation of B cells [11,12] and enhanced CD154-CD40 interactions [13]. Useful insight into the pathogenesis of lupus has been obtained with animal models. MRL/lpr mice spontaneously develop a lupus-like autoimmune disease in an age-dependent manner, including autoantibody production, arthritis, skin lesions, and severe nephritis, which usually leads to early demise from renal failure [14]. When rendered B cell deficient, they no longer develop nephritis, mononuclear infiltrates are no longer detectable in the kidneys or skin, the number of activated memory T cells are markedly reduced, and infusions of pooled serum from diseased MRL/lpr mice lead to glomerular antibody deposition, but not the development of renal disease [15,16]. However, when reconstituted with B cells not able to secrete circulating antibodies, they develop nephritis and vasculitis [17]. As such, it appears that B cells play a direct role in promoting disease beyond the production of autoantibodies [18].

Depleting B cells with anti-CD20 monoclonal antibodies has emerged as a potentially new therapeutic strategy for certain autoimmune diseases. The chimeric monoclonal antibody rituximab depletes B cells by targeting the pan-B cell surface antigen CD20. Preliminary experience with rituximab in about 100 patients with SLE (recently reviewed in [7]) and other autoimmune diseases has been encouraging [6,19-22].

Due to the central role of B cells in the pathogenesis of certain autoimmune diseases, targeted anti-B cell immunotherapies would be expected to offer therapeutic value in the setting of SLE. In addition to CD20, another unique target is CD22, a 135 kDa glycoprotein that is a B-lymphocyte-restricted member of the immunoglobulin superfamily, and a member of the sialoadhesin family of adhesion molecules that regulate B cell activation and interaction with T cells [23-27]. CD22 has seven extracellular domains and is rapidly internalized when cross-linked with its natural ligand, producing a potent co-stimulatory signal in primary B cells [25,28-30]. The function of CD22 in cell signaling is suggested by six tyrosine and three inhibitory domain sequences in the intra-cellular cytoplasmic tail. These inhibitory domains are phosphorylated by the non-receptor kinase Lyn upon B cell antigen receptor (BCR) activation by IgM ligation, leading to the activation and recruitment of SHP-1 phosphatase [31,32]. SHP-1 is a tyrosine phosphatase that negatively regulates several intracellular signaling pathways, including the calcium pathway, through dephosphorylation of signaling intermediates, such as Lyn and Syk. CD22 is first expressed in the cytoplasm of pro-B and pre-B cells, and then on the surface of B cells as they mature, with expression ceasing with B cell differentiation into plasma cells [23]. Studies in CD22-deficient mice and in CD22-negative cell lines have shown an increase in calcium response to BCR ligation [33-36], indicating that CD22 inhibition of BCR signaling is achieved through the mechanism of controlling calcium efflux in B cells. It has been reported that this effect of CD22 is mediated by potentiation of plasma membrane calcium-ATPase and requires SHP-1 [37]. Animal experiments indicate that CD22 plays a key role in B cell development and survival, with CD22-deficient mice having reduced numbers of mature B cells in the bone marrow and circulation, and with the B cells also having a shorter life span and enhanced apoptosis [31].

Therefore, CD22 is an attractive molecular target for therapy because of its restricted expression; it is not exposed on embryonic stem or pre-B cells, nor is it normally shed from the surface of antigen-bearing cells. Initially, a mouse monoclonal antibody (mLL2, formerly EPB-2) was developed and characterized that specifically binds to the third domain of CD22 [38,39]. Immunohistological evaluation revealed that it recognized B cells within the spleen and lymph nodes, but did not react with antigen unrelated to B cells in normal and solid tumor tissue specimens, and flow cytometry showed no reactivity with platelets, red blood cells, monocytes, and granulocytes in normal peripheral blood [38,39]. The complementarity-determining regions of mLL2 were subsequently grafted onto a human IgG_1 genetic backbone [40]. Epratuzumab, the resulting complementarity-determining region-grafted (recombinant) 'humanized' monoclonal antibody (hLL2), is 90% to 95% of human origin, thus greatly reducing the potential for immunogenicity. Epratuzumab has been shown to mediate antibody-dependent cellular cytotoxicity *in vitro*[41] , and may also exhibit biological activity through modulating BCR function (J Carnahan, R Stein, Z Qu, K Hess, A Cesano, HJ Hansen, DM Goldenberg, manuscript submitted).

In clinical trials, over 400 patients with non-Hodgkin lymphoma (NHL) or other B cell malignancies have received epratuzumab administered as 4 consecutive weekly infusions over about 60 minutes. An initial phase I/II study administered doses of up to 1,000 mg/m^2, with patients premedicated each week with oral acetaminophen and diphenhydramine to minimize potential infusion reactions. Epratuzumab toxicity consisted primarily of mild to moderate transient infusion-related events during the first infusion, and only one patient with a prior right lung resection for a fungal abscess had a serious event (bronchospasm during infusion), which was treated with parenteral medications. Based on this safety record, objective evidence of tumor

response, and less severe depression of circulating B cells [42,43], 4 consecutive weekly doses of 360 mg/m^2 epratuzumab was selected as a sufficiently safe and efficacious treatment regimen to warrant further clinical development. A pharmacokinetic analysis of weekly dosing subsequently demonstrated that the post-treatment serum half-life of epratuzumab in NHL patients was 19 to 25 days, consistent with the half-life of a human IgG_1 [44]. As such, a longer interval between doses was indicated, and a biweekly dosing schedule was selected for this initial study in SLE. We report here the first experience of treating an autoimmune disease with a CD22 antibody, epratuzumab.

Materials and methods

This initial, phase II, open-label, non-randomized, single-center study was undertaken to obtain preliminary evidence of therapeutic activity in SLE, to confirm the safety, tolerance and lack of immunogenicity of epratuzumab in this population, and to evaluate pharmacokinetic and pharmacodynamic parameters. The study was approved by the Ethics Committee of Charité University Hospital.

Patient population

Males or non-pregnant, non-lactating females, ≥ 18 years of age, were eligible to participate provided they had a diagnosis of SLE according to the American College of Rheumatology revised criteria (fulfilled ≥ 4 criteria), with SLE for at least 6 months, and at least one elevated autoantibody level (antinuclear antibodies/ANA and/or anti-dsDNA) and moderately active disease (a score of 6 to 12 for total British Isles Lupus Assessment Group (BILAG) disease activity) at study entry. Patients were excluded if they had prior rituximab or other antibody therapy, allergies to murine or human antibodies, experimental therapy within 3 months, active severe CNS (central nervous system) lupus, laboratory abnormalities (hemoglobin < 8.0 g/dl, WBC (white blood cells) < 2,000/mm^3, ANC (absolute neutrophil cells) < 1,500/mm^3, platelets < 50,000/μl, liver transaminases or alkaline phosphatase more than twice upper limit of normal, serum creatinine > 2.5 mg/dl, or proteinuria > 3.5 gm/day), thrombosis, drug or alcohol abuse, infection requiring hospitalization within 3 months, long-term active infectious diseases (tuberculosis, fungal infections) within 2 years, malignancy (except basal cell carcinoma, cervical carcinoma in situ (CIS), history of recurrent abortions (2 or more), or known HIV, hepatitis B or C, or other immunosuppressive states.

Concomitant medications

Pulsed methylprednisolone, other high-dose corticosteroids, cyclophosphamide, and intravenous, joint, or intramuscular corticosteroid injections were not allowed during the study or within four weeks of study entry. Low-dose corticosteroids (prednisone, = 20 mg/day or equivalent) or background therapy with standard antirheumatic immunosuppressives (for example, azathioprine, methotrexate) was permitted provided there were no dosing changes during the study or within four weeks prior to study entry. Antimalarials, non-steroidal anti-inflammatory drugs (NSAIDs), ACE-inhibitors or angiotensin receptor antagonists were also allowed, provided there were no dosing changes during the study or within two weeks of study entry.

Treatment schedule

After satisfying eligibility, signing informed consent, and undergoing baseline evaluations, all patients received 4 doses of 360 mg/m^2 epratuzumab administered every other week with paracetamol (acetaminophen) and an antihistamine (but no steroids) given as premedication prior to each dose.

Study evaluations

The BILAG system was used to categorize the severity level of lupus disease activity in each patient at study entry and at post-treatment evaluations obtained at 6 (24 hours after the last infusion), 10 and 18 weeks and at an additional 32 weeks (6 month post-treatment) follow-up visit. The BILAG system organizes lupus-associated signs and symptoms according to eight body systems: general/constitutional, mucocutaneous, neurological, musculoskeletal, cardiovascular/respiratory, vasculitic, renal, hematological domains [45,46]. At each evaluation, the presence and change of any signs and symptoms were recorded and the level of any disease activity within each body system determined on a treatment-intent basis, according to BILAG rules as: A (severely active disease sufficient to require disease-modifying treatment, for example, > 20 mg/d prednisolone, immunosuppressants/cytoxics); B (moderately active disease requiring only symptomatic therapy, for example < 20 mg/d prednisolone, antimalarials, NSAIDs alone or in combination); or C (stable mild disease with no indication for changes in treatment). To assign an overall disease activity level for each patient, a total BILAG score was determined by adding a numerical severity score (A = 9, B = 3, C = 1, no activity = 0) across the eight body systems. Other evaluations at these times included an SLE panel (autoantibodies, C3, C-reactive protein/CRP, erythrocyte sedimentation rate/ESR, other laboratory tests), vital signs, physical examination, adverse events, routine safety laboratory tests (hematology, serum chemistry), urinalysis, serum immunoglobulins, peripheral blood B and T cells, epratuzumab serum levels (analyzed by sponsor), and human anti-human (epratuzumab) antibody titers (HAHA; analyzed by sponsor).

Human anti-human (epratuzumab) antibody assay

The sponsor's HAHA test is a competitive ELISA assay, where the capture reagent is epratuzumab and the probe is an anti-epratuzumab-idiotype antibody. The anti-idiotype antibody is an acceptable surrogate for what is reacted against in an immunogenic response by humans against the binding portion of epratuzumab that distinguishes the molecule from other human antibodies (for instance, the framework region that has human amino acid sequences). Test results are derived from

Table 1

Number of patients with B-level disease activity at study entry in each BILAG body system

Body system	Number of patients	Contributing signs/symptoms* (number of patients)
I. General/ constitutional	3	Fatigue/malaise/lethargy (3)
		Anorexia/nausea/vomiting (2)
		Unintentional weight loss > 5% (1)
II. Mucocutaneous	13	Malar erythema (11)
		Active localized discoid lesions (2)
		Mild maculopapular eruption (1)
III. Neurological	0	
IV. Musculoskeletal	2	Arthritis (2)
V. CV/Respiratory	2	Dyspnea (2)
		Pleuropericardial pain (2)
VI. Vasculitis	5	Minor cutaneous vasculitis (nailfold/digital vasculitis, purpura, urticaria) (5)
VII. Renal	0	
VIII. Hematology	1	Anemia (hemoglobin < 11 g/dL) (1)

*Signs and symptoms that contributed to the B-level disease activity according to BILAG rules.

an eight-point standard curve with varying dilutions of anti-idiotype antibody in bovine serum albumin. Patient serum samples are diluted 1:2 with bovine serum albumin and assayed in triplicate. The anti-idiotype standard curve is used to determine the presence of HAHA in unknown samples. An acceptable assay is based on linear regression parameters that must be met to define a valid assay.

Statistical analyses

The primary assessment of disease activity compared post-treatment BILAG results with those at study entry, using total BILAG scores for overall assessment and letter grade categories to assess the level of disease activity within each body system. Adverse events and safety laboratory tests were graded according to NCI CTC version 3.0 criteria on a 1 to 4 scale for toxicity (1, mild; 2, moderate; 3, severe; 4, life threatening). All analyses of efficacy, safety, tolerance, immunogenicity, pharmacokinetics, and pharmacodynamics used descriptive statistics. Wilcoxon signed rank test was used to assess the statistical significance of changes in total BILAG scores compared to their baseline values. All statistical tests used a significance level of 0.05.

Table 2

Number of patients with C-level disease activity at study entry in each BILAG body system

Body system	Number of patients	Contributing signs/symptoms* (number of patients)
I. General/ Constitutional	11	Fatigue/malaise/lethargy (10)
		Anorexia/nausea/vomiting (1)
		Lymphadenopathy/splenomegaly (1)
		Pyrexia (documented) (1)
II. Mucocutaneous	1	Mild alopecia (1)
III. Neurological	10	Episodic migrainous headaches (8)
		Severe, unremitting headache (2)
IV Musculoskeletal	11	Arthralgia (10)
		Myalgia (9)
		Improving arthritis (1)
V. CV/ Respiratory	2	Dyspnea (1)
		Pleuropericardial pain (1)
VI. Vasculitis	4	Raynaud's (3)
		Livido reticularis (1)
VII. Renal	4	Mild/stable proteinuria (4)
VIII. Hematology	11	Lymphocytopenia (< 1500 cells/μl) (10)
		Evidence of circulating anticoagulant (1)
		Decreased platelets (< 150,000/μl) (1)

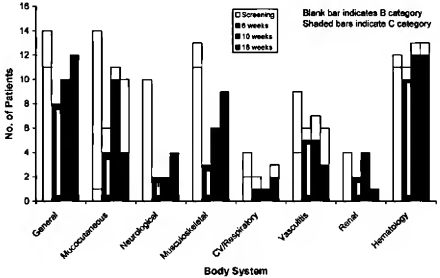
*Signs and symptoms that contributed to the C-level disease activity according to BILAG rules.

Results

Demographics and patient characteristics at study entry

A total of 14 Caucasian patients (13 females and 1 male; 23 to 53 years old, median age 40 years) were enrolled. At study entry, the patients had been initially diagnosed with SLE 1 to 19 years (median 10 years) earlier and were receiving corticosteroids ($n = 13$, 1 to 12 mg/day prednisolone) plus immunosuppressives ($n = 11$, including 50 to 200 mg/day azathioprine, $n = 9$; 20 mg/day methotrexate, $n = 2$; 2 g/day mycophenalate mofetil, $n = 1$), and antimalarials ($n = 6$, 200 to 600 mg/day hydroxychloroquine). All patients had positive ANA at study entry (titers of 80:1 to 5,120:1), and 5 patients (36%) had positive anti-dsDNA antibody levels (> 10 U/ml). Ten patients (71%) had ESR values that were elevated (> 15 mm/h) and 4 patients (29%) had raised CRP levels (> 0.5 mg/dl), while only 3 patients (21%) had C3 levels that were borderline low or decreased (< 90 mg/dl), and no patient had

Figure 1



Frequency comparison of BILAG B- and C-level activities for each body system at screening, 6, 10 and 18 weeks.

positive direct Coombs' or serum haptoglobulin levels elevated above borderline.

All patients had total BILAG scores of 6 to 12 (median 10) at study entry. No patient had A-level disease activity in any body system, 13 patients had B-level disease activity in at least one body system (2 with three Bs, 9 with 2 Bs, 2 with one B) and one patient had only C-level activities. B-level disease occurred primarily in the mucocutaneous, vasculitis, and general/constitutional body systems, with no B-level disease activity in the neurological or renal systems (Table 1), while C-level disease occurred primarily in the general/constitutional, musculoskeletal, hematological and neurological body systems (Table 2). The actual signs and symptoms at study entry that contributed to the B-level disease activity according to the BILAG rules are also summarized in Table 1, while those contributing to C-level disease activity are summarized in Table 2.

Study drug administration

Twelve of the 14 patients (86%) completed all 4 infusions of 360 mg/m² epratuzumab as scheduled, while one patient with sleepiness attributed to premedication IV antihistamines prematurely terminated the first infusion but subsequently completed all 3 remaining infusions without further event, and one patient completed the first two infusions, but discontinued further infusions after development of herpes zoster, which responded to antivirals. The infusions were well tolerated, with a median infusion time of 32 minutes (23 to 86 minutes), and with infusion reactions in 6 patients all limited to occurrences of transient, mild (grade 1 NCI toxicity) adverse events (flu-like symptoms, tracheitis/throat ache, $n = 2$; arthralgia/myalgia, fever, fatigue, nausea, headache, chills, or rash, $n = 1$).

Post-treatment evaluations and follow-up

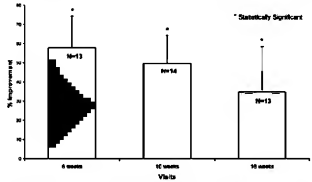
All patients remained in the study through the 18-week post-treatment evaluation period. One patient had a late 18-week visit that fell within the 32-week time frame and the corresponding data were hence re-assigned to the 32-week visit. The single patient who did not complete all 4 infusions continued to receive post-treatment evaluations beginning at the 10-weeks follow-up visit. Except for the aforementioned deviations, all patients received post-treatment evaluations at 6, 10, and 18 weeks. One patient was lost to follow-up after 18 weeks, while 13 patients returned for the final 32-week evaluations (8 patients as scheduled, 5 with a delayed visit between 42 to 82 weeks).

BILAG treatment response

The effect of epratuzumab on clinical manifestations was evaluated at 6, 10, and 18 weeks using numerical total BILAG scores as well as categorical scores. The compositions of B- and C-level activities improved after treatment, primarily in the general, mucocutaneous and musculoskeletal systems (Figure 1). Improvement in C-level activity was also observed in the neurological and renal domains. Improvements in the general, mucocutaneous, neurological and musculoskeletal systems occurred earlier compared to the cardiovascular/respiratory, vasculitic and renal systems (Figure 2). However, the limited number of patients with manifestations in each of these systems precludes a definitive determination of preferential effects. In terms of changes in the total BILAG score, statistically significant improvement was observed at 6, 10, and 18 weeks (Figure 3). Additionally, a substantial proportion of patients showed 50% or more improvement in total BILAG score at weeks 6, 10, and 18 (77%, 71% and 38%, respectively). At the final 32-week evaluation, statistically significant

Figure 2

Level of Improvement	6 weeks	10 weeks	18 weeks
Pts with Decreased Scores	100% (13/13)	100% (14/14)	92% (12/13)
Pts with Decreases ≥ 50%	77% (10/13)	71% (10/14)	38% (5/13)



Overall frequency and mean improvement of total disease activity as measured by the total BILAG score at 6, 10 and 18 weeks.

improvement in total BILAG score continued to be observed, with 15% of the patients achieving 50% or more improvement.

In a separate analysis, the total number of patients who achieved BILAG improvements in the particular domains at 6, 10 and 18 weeks of follow-up are summarized in Table 3. This indicates that the most characteristic BILAG domains, as also seen in Figure 2, were more likely to respond, although the duration of response was very similar throughout the domains. In fact, deterioration in BILAG categorical scores compared to baseline was infrequently seen during the study (Table 4). Only two patients (14%) showed worsening of hematological

Table 3

Number of patients with improvement from baseline BILAG B- and C-level activities

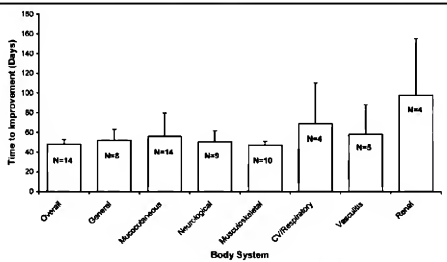
BILAG body system	6 weeks[a]	10 weeks	18 weeks
General ($N = 14$)[b]	6 (43%)	5 (36%)	2 (14%)
Mucocutaneous ($N = 14$)	11 (79%)	8 (57%)	6 (43%)
Neurological ($N = 10$)	7 (70%)	8 (80%)	6 (60%)
Musculoskeletal ($N = 13$)	9 (69%)	7 (54%)	4 (31%)
CV/Respiratory ($N = 4$)	3 (75%)	3 (75%)	3 (75%)
Vasculitis ($N = 9$)	4 (44%)	3 (33%)	3 (33%)
Renal ($N = 4$)	2 (50%)	1 (25%)	3 (75%)
Hematology ($N = 12$)	0 (0%)	0 (0%)	0 (0%)
Overall[c] ($N = 14$)	13 (93%)	14 (100%)	13 (93%)

[a]Twenty-four hours after fourth infusion. [b]N = number of patients with involvement in a particular body system at entry. [c]As applied to any BILAG body system.

parameters (lymphocytopenia), one starting at 6 weeks and the other at 18 weeks. Another patient manifested renal (mild proteinuria) deterioration at 10 weeks. Overall, at week 18, 3 patients (21%) had a deteriorated BILAG assessment in at least one body system compared to baseline.

An additional analysis was performed to determine the durability of resolution of certain B- and C-level activities (Table 5). Although in a number of patients, B- and C-level activities resolved persistently, the heterogeneity of patients' manifesta-

Figure 3



Mean time to improvement of each BILAG body stystem. Mean time to improvement (in days) of each BILAG body system during the follow-up of the study (N denotes the number of patients available for analysis for each body system). Since the first evaluation was scheduled for 6 weeks, the earliest time to improvement is at least 42 days.

tions again precluded the identification of a preferential response profile to the drug.

Safety

During or following treatment, a total of ten patients reported adverse events. As reported above, six had mild, transient, infusional reactions and one patient experienced somnolence following antihistamine medication. Subsequently, five patients had infections (including herpes zoster, otitis media, *Helicobacter pylori*-associated gastritis, vaginitis/vaginal candidiasis, cystitis, and tonsillitis) that resolved with appropriate treatment, and one patient had spinal contusion from a traffic accident. Standard safety laboratory tests showed no consistent pattern of change from baseline, and infrequent post-treatment increases in NCI CTC v3.0 toxicity grades for these laboratory tests were all limited to changes of one grade level except for one patient with an increase in lymphocytes from grade 1 to grade 3, and another from grade 0 to grade 3 (Table 6).

Pharmacokinetics and immunogenicity

Of the 14 patients, serum samples for analysis of pharmacokinetics and immunogenicity (HAHA) by ELISA assay were collected in a limited number of patients post-treatment at 6 weeks ($n = 12$), 10 weeks ($n = 7$) and 18 weeks ($n = 7$). Epratuzumab serum levels were measurable in all available samples through at least 10 weeks post-treatment and were still detectable above the 0.5 μg/ml assay limit in 5/7 samples evaluated at 18 weeks, with median values of 120 μg/ml (range 49 to 350) at 6 weeks, 48 μg/ml (range 31 to 138) at 10 weeks, and 8.3 μg/ml (range 1.82 to 25) at 18 weeks. Figure 4 shows the individual measurements over time. There was a single sample showing 1.42 μg/ml at 32 weeks. HAHA analysis gave no evidence of immunogenicity, with all post-treatment values either remaining below the 50 ng/ml sensitivity of the assay or not increased from baseline values prior to treatment.

Immunology laboratory tests

Table 7 shows that at the first evaluation after treatment, mean B cell levels decreased by 35% and persisted at these levels on subsequent evaluations (Figure 5), with no evidence of onset of recovery by the final study evaluation at 32 weeks (6 months post-treatment). In contrast, there does not appear to be any consistent pattern of decreases/increases in T cell levels or serum levels of IgG, IgA, or IgM following treatment (Table 7).

Although all 14 patients had measurable ANA titers (1:80 to 1:5,120) at study entry, no patient had consistent post-treatment decreases, including evaluations at 32 weeks (6 months post-treatment) follow-up (8 patients had no changes at any evaluation, 5 doubled their baseline titers at one or more evaluations, and one patient had an isolated decrease at one evaluation). Five patients had elevated anti-dsDNA antibodies (10 to 123 U/ml) at study entry, but none had any decreased post-treatment values (2 patients had no significant changes, and 3 had increases at one or more evaluations). C3 levels that were decreased or borderline for 3 patients at study entry remained virtually unchanged post-treatment, as did mean C3 values for all patients.

Table 4

Number of patients with deteriorating BILAG activities from baseline

BILAG body system ($N = 14$)[a]	6 weeks[b]	10 weeks	18 weeks
General	0 (0 %)	0 (0 %)	0 (0 %)
Mucocutaneous	0 (0 %)	0 (0 %)	0 (0 %)
Neurological	0 (0 %)	0 (0 %)	0 (0 %)
Musculoskeletal	0 (0 %)	0 (0 %)	0 (0 %)
CV/Respiratory	0 (0 %)	0 (0 %)	1 (7 %)
Vasculitis	0 (0 %)	0 (0 %)	0 (0 %)
Renal	0 (0 %)	1 (7 %)	0 (0 %)
Hematology	1 (7 %)	1 (7 %)	2 (14%)
Overall[c]	1 (7 %)	2 (14%)	3 (21%)

[a]N = total number of patients. [b]Twenty-four hours after fourth infusion. [c]As applied to any BILAG body system.

Table 5

Number of patients in each BILAG body system with resolution of baseline B- and C-level disease activities

Body system	B level	C level
General	3/3 (100%)	0/11 (0%)
Mucocutaneous	4/13 (31%)	0/1 (0%)
Neurological	0/0	2/10 (20%)
Musculoskeletal	1/2 (50%)	1/11 (9%)
CV/Respiratory	0/2 (0%)	2/2 (100%)
Vasculitis	2/5 (40%)	0/4 (0%)
Renal	0/0	2/4 (50%)
Hematology	0/1 (0%)	0/11 (0%)

Resolution is defined as post-treatment improvement of baseline disease activity level by at least one category level (B to C, D, or E; C to D or E) at one or more evaluations up to 18 weeks, with no categorical deterioration from the baseline activity level prior to improvement, and no reversion to the baseline activity level once any improvement has occurred. Additionally note that 3 patients with multiple BILAG B involvement at baseline had completely resolved all B-level disease activities by 18 weeks.

Table 6

Post-treatment increases in NCI CTC v3.0 toxicity grades from baseline values

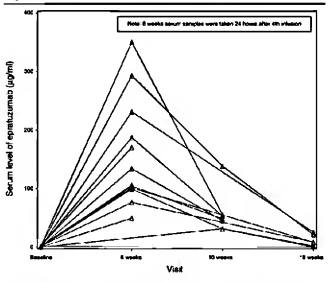
Labparameter	No increase	Toxicity increase	
		1 grade	2–3 grades
Hematology			
Hemoglobin	10	4	0
Platelets	12	2	0
WBC	11	3	0
ALC	6	6	2
ANC	13	1	0
Chemistry			
Creatinine	10	4	0
Total Bilirubin	14	0	0
Alkaline phosphatase	12	2	0
ALT (SGPT)	9	5	0
AST (SGOT)	10	4	0
GGT	12	2	0

ALC, absolute lymphocyte count, ANC, absolute neutrophil count, ALT, alanine aminotransferase, AST, aspartate aminotransferase, GGT, gamma glutamyl transferase, WBC, white blood cell

Discussion

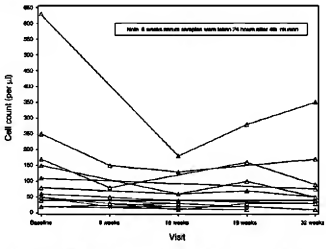
The pathogenesis of SLE remains enigmatic, but a central feature of this disease is the loss of immune tolerance and enhanced B cell activity. Although the number of B cells in the peripheral blood is often decreased, those that are present show characteristic alterations and have abnormal phenotypes indicative of activation [5,47]. Therefore, B cell depletion is an attractive therapeutic strategy for patients with SLE. The availability of the chimeric anti-CD20 antibody rituximab (Rituxan® Genentech, South San Francisco, CA, USA; Biogen Idec, Boston, MA, USA) made it possible to test this hypothesis.

Figure 4



Serum levels of epratuzumab as detected by ELISA in the patients during the study.

Figure 5



Follow-up of peripheral B cell levels during the study among individual study patients.

Initially, Isenberg and coworkers [19] treated 6 patients with active and otherwise refractory SLE (median BILAG score 14, range 9 to 27) with rituximab given in 500 mg doses 2 weeks apart with 2 doses of 750 mg iv cyclophosphamide and oral prednisolone cover (30 or 60 mg for 5 days). The treatment was safe and well tolerated, B cell depletion occurred, and BILAG total scores improved at 6 months (median 6, range 3 to 8). Looney and colleagues [6] initiated an open-label rituximab study of 17 patients with SLE (≥ 6 systemic lupus activity measurement, SLAM score) who were treated with either one 100 mg/m^2 dose, one 375 mg/m^2 dose, or four 375 mg/m^2 doses. Oral prednisone (40 mg for two doses) also was administered. B cell decreases were variable, with a 35% mean decrease persisting over the 6-month observation period, and clinical efficacy was demonstrated in patients with B cell depletion. Less than 6/17 of their patients developed human anti-chimeric antibody (HACA) at a level higher than or equal to 100 ng/ml when treated with this protocol.

All of these studies and case reports have so far been of short duration [7,48]. Usually, the B cell depletion in SLE is profound, as in patients with NHL, but shorter lasting. Therefore, it is very likely that cyclical therapy will be needed to provide long-term benefit for patients with SLE. While the immunogenicity of rituximab has not been clinically important (HACA < 1%) for the management of patients with NHL, approximately 4% of patients with rheumatoid arthritis developed HACA and 8% to 10% with SLE did so also, in spite of being

Table 7

Post-treatment changes of lymphocytes and immunoglobulins

	Baseline values and post-treatment percent change from baseline (mean ± SD)				
	Baseline	6 weeks	10 weeks	18 weeks	32 weeks
Lymphocytes	$N = 14$	$N = 6$	$N = 8$	$N = 9$	$N = 11$
B cells	123 ± 160 cells/μl	-35% ± 23%	-41% ± 41%	-34% ± 23%	-44% ± 21%
T cells	744 ± 554 cells/μl	+16% ± 80%	+28% ± 78%	+47% ± 109%	+17% ± 69%
Immunoglobulins		$N = 12$	$N = 14$	$N = 10$	$N = 11$
IgG	1,252 ± 355 mg/dl	+3% ± 8%	+5% ± 13%	+5% ± 9%	1% ± 13%
IgA	226 ± 94 mg/dl	+3% ± 11%	+8 ± 13%	+5% ± 12%	+10% ± 20%
IgM	117 ± 73 mg/dl	-12% ± 18%	-1% ± 23%	-6% ± 19%	-9% ± 9%

SD, standard deviation.

treated with various doses of steroids and/or cytotoxic agents in combination with rituximab. Thus, a less immunogenic antibody (for example, a human or humanized form) is likely needed in the management of patients with autoimmune diseases, since it is expected that repeated dosing will be required in patients with such chronic diseases.

This initial study demonstrated that 360 mg/m^2 epratuzumab, a humanized CD22-specific monoclonal antibody, administered every other week for a total of 4 doses was safe and well-tolerated in SLE patients, with few significant adverse events, alterations of standard safety laboratory tests, and no evidence of immunogenicity. In addition to the minimal infusion reactions, the ability to complete an infusion within approximately 0.5 to 1 hour and the lack of immunogenicity are also likely to be more important treatment considerations in autoimmune diseases, as mentioned previously.

With this dosing schedule, virtually every patient with moderate disease activity (total BILAG score of 6 to 12) demonstrated symptomatic improvement using BILAG total scores. The BILAG total score results indicate that 77% of the patients achieved a ≥ 50% decrease in their overall disease activity at 6 weeks follow up. Furthermore, most patients (92%) continued to show reduced disease activity for at least 18 weeks, and even 38% showed a sustained response with BILAG reductions of 50% or more compared to study entry. Since this first study considered moderately active lupus patients with BILAG total scores of 6 to 12, the resulting heterogeneity precludes the identification of any preferential effect on one or the other BILAG domains as shown from different perspectives of efficacy analysis.

In addition to treating mild BILAG C-level symptoms, epratuzumab immunotherapy reduced all BILAG B-level activity in the majority of patients presenting with more serious disease, including patients with B-level activity in several body systems. The current data limit the conclusions that can be drawn regarding therapeutic effects for some systems, such as B-level disease in the neurological and renal systems, and only one case of lymphopenia in the hematological system showed improvement. In spite of small numbers, CD22-immunotherapy with epratuzumab appeared to be effective for treating disease in many of the other body/organ systems.

Although the biweekly dosing schedule used in this study demonstrated apparent activity, the serum levels of antibody measured here appear to be less than those in studies of NHL, where a weekly schedule of dose administrations has shown antitumor activity [42-44]. Hence, other dosing schedules in future clinical trials are warranted to assess the effects of increasing the serum levels of epratuzumab.

Compared to the complete depletion of B cells observed with rituximab, a long-lasting (at least 6 months, the last observation time) decrease of about 35% to 40% occurred with epratuzumab, with no apparent changes in T cells or immunoglobulin levels. As discussed earlier, the attractiveness of CD22 as a molecular target for therapy in SLE extends beyond the capability of epratuzumab to modestly decrease peripheral blood levels of B cells. CD22 is a cell surface receptor that is a member of the sialioadhesion family and an inhibitory co-receptor of BCR [34]. *In vitro* studies demonstrated that epratuzumab binding can induce CD22 phosphorylation [49] , and the current data from this study suggest that epratuzumab could potentially mediate direct pharmacological effects by negatively regulating certain hyperactive B cells. This hypothesis now needs to be tested. Interestingly, over the period of this study, patients clinically improved without clear evidence of reduction in ANA or anti-dsDNA titers. Similar observations have been reported with rituximab [19] , further supporting the hypothesis that targeted therapy impacting the hyperactive B cell compartment may be successful without needing to completely deplete the broader B cell population.

Conclusion

This initial experience in lupus patients with mild to moderate symptoms demonstrated that 4 doses of 360 mg/m^2 epratuzumab immunotherapy are safe and well tolerated when infused within one hour, with consistent improvement observed in all patients for at least 12 weeks in the presence of modestly decreased (about 35%) peripheral B cell levels, and with no evidence of HAHA. Although this was an open-label study, consistent improvement was observed in all patients for at least 12 weeks, and there was reduction or elimination of disease activity across most body systems, regardless of the extent or the severity of the presenting disease activity. The duration of response was very heterogeneous for different BILAG domains, precluding firm conclusions at this time. As such, these results support conducting longer-term multicenter randomized controlled studies, which are now underway to examine the effects of epratuzumab in broader patient populations with autoimmune disease.

Competing interests

TD, JK, and GRB declare research funding for this study provided by Immunomedics, Inc. WAW, NT, and DMG have employment and financial interests (stock) in Immunomedics, Inc., whichowns the antibody tested in this paper.

Authors' contributions

All authors contributed to data interpretation and the final manuscript. TD and GRB were the principal investigators and were responsible for coordinating the study, while JK participated in patient selection and directed all patient related study procedures. DMG, TD and WAW designed the clinical trial protocol, and NT was responsible for data management and statistical analysis. TD and JK contributed equally to this work.

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Research article

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Epratuzumab (humanised anti-CD22 antibody) in primary Sjögren's syndrome: an open-label phase I/II study

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Abstract

This open-label, phase I/II study investigated the safety and efficacy of epratuzumab, a humanised anti-CD22 monoclonal antibody, in the treatment of patients with active primary Sjögren's syndrome (pSS). Sixteen Caucasian patients (14 females/2 males, 33–72 years) were to receive 4 infusions of 360 mg/m^2 epratuzumab once every 2 weeks, with 6 months of follow-up. A composite endpoint involving the Schirmer-I test, unstimulated whole salivary flow, fatigue, erythrocyte sedimentation rate (ESR), and immunoglobulin G (IgG) was devised to provide a clinically meaningful assessment of response, defined as a ≥20% improvement in at least two of the aforementioned parameters, with ≥20% reduction in ESR and/or IgG considered as a single combined criterion. Fourteen patients received all infusions without significant reactions, 1 patient received 3, and another was discontinued due to a mild acute reaction after receiving a partial infusion. Three patients showed moderately elevated levels of Human anti-human (epratuzumab) antibody not associated with clinical manifestations. B-cell levels had mean reductions of 54% and 39% at 6 and 18 weeks, respectively, but T-cell levels, immunoglobulins, and routine safety laboratory tests did not change significantly. Fifty-three percent achieved a clinical response (at ≥20% improvement level) at 6 weeks, with 53%, 47%, and 67% responding at 10, 18, and 32 weeks, respectively. Approximately 40%–50% responded at the ≥30% level, while 10%–45% responded at the ≥50% level for 10–32 weeks. Additionally, statistically significant improvements were observed in fatigue, and patient and physician global assessments. Further, we determined that pSS patients have a CD22 over-expression in their peripheral B cells, which was downregulated by epratuzumab for at least 12 weeks after the therapy. Thus, epratuzumab appears to be a promising therapy in active pSS, suggesting that further studies be conducted.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease with a population prevalence of approximately 0.5% [1]. The lymphoid infiltrates within the inflamed tissues contain ectopic germinal center-like structures in 20% of patients [2]. These structures consist of T- and B-cell aggregates containing proliferating lymphocytes, follicular, dendritic, and activated endothelial cells [3]. B-cell homeostasis is disturbed in pSS, with diminished frequencies and absolute numbers of peripheral CD27$^+$ memory B cells. However, the infiltrating B cells are mainly CD27$^+$ memory B cells and CD27^{high} plasma cells [4-6]. This altered B-cell subtype recirculation from inflamed tissue was confirmed recently by Hansen *et al.* [7]. Thus, although pSS is considered to be a T-cell-mediated disease, high levels of B-cell autoreactivity have been associated with high disease activity, the development of systemic com-

ACR = American College of Rheumatology; AE = adverse event; ANA = antinuclear antibody; APC = allophycocyanin; AT = artificial tear; BCR = B-cell antigen receptor; CRP = C-reactive protein; CTC = [National Cancer Institute] Common Toxicity Criteria; ELISA = enzyme-linked immunosorbent assay; ESR = erythrocyte sedimentation rate; HACA = human anti-chimeric antibody; HAHA = human anti-human (epratuzumab) antibody; Ig = immunoglobulin; NHL = non-Hodgkin's lymphoma; PE = phycoerythrin; pSS = primary Sjögren's syndrome; RF = rheumatoid factor; SD = standard deviation; SLE = systemic lupus erythematosus; USF = unstimulated whole salivary flow; VAS = visual analogue scale.

plications, and an increased risk of development of B-cell lymphoma [8].

This has led to anti-B-cell monoclonal antibody immunotherapy emerging as a promising new treatment modality in pSS and autoimmune disorders [9]. The use of rituximab, a chimeric anti-CD20 antibody, has been reported in small studies and case reports of SS patients with or without associated lymphoma [10-13]. However, serum sickness-like diseases seem to occur in approximately 20% of patients treated with this chimeric antibody [11] and may be of major clinical concern in subjects with a hyperactive immune system.

CD22 is a 135-kDa B-lymphocyte restricted type-I transmembrane sialoglycoprotein of the immunoglobulin (Ig) superfamily, with seven Ig-like domains and three cytoplasmic ITIMs (immunoreceptor tyrosine-based inhibitory motifs) [14]. CD22 appears intracellularly during the late pro-B-cell stage of ontogeny, shifting to the plasma membrane with B-cell maturation. CD22 is expressed at low levels on immature B cells, expressed at higher levels on mature IgM^+, IgD^+ B cells, and absent on differentiated plasma cells. It is strongly expressed in follicular, mantle, and marginal-zone B cells but is weakly present in germinal B cells (reviewed in [15]). The function of CD22 has not been entirely clarified; it acts as a homing receptor for recirculating B cells through the affinity of the lectin-like domains for 2,6-linked sialic acid-bearing glycans and as a B-cell antigen receptor (BCR) down-modulating coreceptor [16,17]. Because dysregulated expression of CD22 could lead to excessive activation of B cells and autoantibody production [17], targeting this coreceptor in systemic autoimmunity appears to be a potentially new therapeutic pathway. Indeed, antagonistic antibodies to CD22 could provoke downregulation of the BCR (by SHP-1 [Src homology phosphatase-1] recruitment) and inhibiting T- and B-cell crosstalk by downregulation of the CD40 pathway [15,18,19].

Epratuzumab (hLL2), a humanised IgG1 monoclonal antibody directed against the CD22 antigen, binds the third cytoplasmic Ig domain of CD22 [20]. Its known mechanism of action is believed to be the downregulation of the BCR, with mechanisms of action differing from rituximab (by CD22 phosphorylation and BCR effects via immobilised Ig crosslinking) [18,21]. Also, epratuzumab depletes circulating B cells when given to patients with non-Hodgkin's lymphoma (NHL) [22] or systemic lupus erythematosus (SLE) [23], but markedly less than rituximab [24], and has been therapeutically active in both diseases.

Based on these considerations, this phase I/II study was undertaken to investigate the safety and potential local and systemic effects of epratuzumab in patients with active pSS. The effect of this treatment on the peripheral B and T cells in this patient population was also assessed.

Materials and methods

Study design

This phase I-II, open-label, 18-week study was conducted at two centres. The protocol was approved by the local ethics committees, and all patients enrolled were required to provide signed informed consent. The study included four infusions of epratuzumab (360 mg/m^2) at 0, 2, 4, and 6 weeks and three follow-up evaluations at 6, 10, and 18 weeks (that is, 1 day, 4 weeks, and 12 weeks after the fourth infusion). Additionally, a final long-term evaluation was scheduled at 32 weeks (6 months post-treatment).

Patient population

Males or non-pregnant, non-lactating females, at least 18 years of age, were eligible to participate provided that they fulfilled the American/European consensus group classification criteria for pSS [25]. For females of childbearing potential, a negative pregnancy test result and adequate contraception during the study and for 6 months after the last infusion were required. Additionally, all patients had to demonstrate active pSS prior to study entry. Because there are no validated disease activity criteria for pSS, this was defined as increased B-cell activity, IgG greater than 1.4 g/l or erythrocyte sedimentation rate (ESR) greater than 25 mm/hour, in combination with the presence of autoantibodies. In addition, the patients must have been on symptomatic treatment for at least 6 months prior to screening. Disease-modifying drugs, such as hydroxychloroquine, methotrexate, cyclosporin, sulfasalazine, or corticosteroids, were not allowed during the study and were discontinued at least 4 weeks before study entry. No prior treatment with rituximab or other anti-B-cell antibodies was allowed. The exclusion criteria included serious infections in the previous 3 months, documented HIV or hepatitis-B or -C infection, known malignancy, severe or uncontrolled concurrent disease, and the presence of any other autoimmune/connective tissue disease.

Study drug administration

Epratuzumab at the dose of 360 mg/m^2 in 250 ml 0.9% sterile NaCl was prepared by the hospital pharmacy (Erasme University Hospital and Charite Humboldt University Hospital). The total dose was to be given throughout a 40-minute period. To minimise hypersensitivity, patients were premedicated with acetaminophen (0.5–1 g) and antihistamine (25–50 mg *per os* or intravenous polaramin). Four intravenous infusions of epratuzumab were given at 0, 2, 4, and 6 weeks.

Concomitant medications

Patients were allowed to continue artificial tears (ATs) and artificial saliva substitutes or nonsteroidal anti-inflammatory drugs provided that the dosage and schedule regimens were stable for at least 4 weeks and were monitored during the study.

Clinical assessment

Clinical, ophthalmological, and biological evaluations were performed at study entry and at 6, 10, and 18 weeks (that is, 1 day, 4 weeks, and 12 weeks post-treatment). A final evaluation at 32 weeks was also scheduled for patients who were still in long-term follow-up. Clinical assessment (performed by the same physician) consisted of a general physical examination; a dry mouth evaluation (0–2 scale: 0 = none, 1 = mild to moderate, and 2 = severe) involving the collection of unstimulated whole saliva throughout a 15-minute interval by using the spitting technique according to established methods [26] (note: saliva samples were weighed on an analytical balance to determine the volume of saliva obtained, using the conversion formula 1 g = 1 ml); a dry eye evaluation (0–2 scale: 0 = no symptoms, 1 = mild to moderate symptoms relieved by ATs, and 2 = severe symptoms unrelieved by AT); the Schirmer-I test; evaluation of fatigue by a 0–100 mm visual analogue scale(VAS) and a questionnaire (0 = no fatigue, 1 = mild fatigue not interfering with daily activities, 2 = moderate fatigue that interferes with daily activities, and 3 = fatigue with severely reduced activities); the tender/swollen joint count (maximum 36); and the tender point count (maximum 18). The patient's pain assessment and the patient's and physician's global assessments were evaluated by a 0–100 mm VAS. The following biological parameters were measured throughout the study: ESR, C-reactive protein (CRP), complete blood count, renal and liver function tests, creatine phosphokinase, serum Igs (A, M, and G), antinuclear antibodies (ANAs), rheumatoid factor (RF), and peripheral blood B- and T-cell counts.

For purposes of efficacy evaluation, we focused on the main parameters that consisted of the the following parameters: Schirmer-I test, unstimulated whole salivary flow (USF), fatigue VAS, and the laboratory parameters (ESR and IgG). To assess the overall efficacy of epratuzumab in the treatment of pSS, a composite endpoint involving all five parameters was devised to provide a clinically meaningful definition of response. Specifically, a patient was deemed to be a responder if he/she experienced improvement of 20% or more in at least two of the aforementioned parameters, with reduction of at least 20% in ESR and/or IgG contributing jointly as a single combined criterion. Additional assessments of the efficacy data were also performed using improvements of at least 30% and at least 50% in the efficacy parameters.

Determination of B-cell populations by flow cytometry

Monoclonal antibodies used in this study were CD19 allophycocyanin (APC) (clone SJ25C1) and CD22 phycoerythrin (PE) (clone S-HCL-1). The median channel of fluorescence on the 256-channel linear scale was employed to define numerically the fluorescence distribution of CD22 in a semi-quantitative approach. We routinely used a four-color FACScalibur with automatic loader, driven by Cellquest software that was set up using the three-color FACScomp software and Calibrite microbeads (Becton, Dickinson and Company, San Jose, CA, USA). The stability of the fluorescence intensity signal over a long period of time was assessed using Quantum 1000 microbeads (weekly) and daily with the Rainbow calibration beads from Spherotec (Libertyville, IL, USA) without changing the PMT (photomultiplier tube) voltage and compensations. At the beginning of each new lot of beads, we determined an acceptable range by running aliquots of all beads 10 times and calculating the mean ± standard deviation (SD) and the CV (coefficient of variation) for that lot of beads. For each lot, we determined a mean target channel value for monitoring of flow cytometer performance. Between lots, flow cytometer settings were adjusted to restart the monitoring with the same target channel value as before. In a previous study with 35 hematological patients sampled more than three times during their clinical course (with a mean inter-visit interval of 123 days and range of 13–638 days) and a mean duration of the survey of 492 days (range 13–1,022 days), we observed an inter-contact variation of only 6.2% for CD5 (which has a sharp distribution in CLL [chronic lymphocytic leukemia]) and 6.1% for CD20. Considering the low scattering of CD22 expression on normal B-lymphocytes, we determined a range of normal values and the median CD22 fluorescence on 33 blood samples from normal healthy volunteers. Because the distribution of the median intensity is normal, we determined ± 2 SD of the distribution to define the normal median fluorescence values from a normal range between channels 159 and 178.

Tri-color immunophenotyping of B-lymphocytes was performed with predetermined combinations of murine monoclonal or rabbit polyclonal (for the Ig light chain staining) antibodies directly conjugated with fluorescein isothiocyanate, PE, and CD19 APC as a marker for B-lymphocytes. A lysed and washed whole-blood technique was used. In this procedure, 50 µl blood samples were incubated with the Mab combination at room temperature for 15 minutes. The red blood cells were then lysed using 500 µl ammonium chloride lysing solution. Cells gated in the mononuclear area in a forward-versus side-scatter dot-plot and also those present in a region around the CD19-positive side-scatter low events were considered to be B cells.

Safety assessments

During the infusion and for 1 hour afterward, the patients were monitored for adverse reactions and vital signs (blood pressure, pulse, and temperature) every 30 minutes. At each visit, patients were asked about any adverse events (AEs) that they experienced. Analysis of pharmacokinetics consisted of epratuzumab levels measured at 30 minutes prior to and after each infusion and at 6, 8, 9, 10, 14, and 18 weeks. Human anti-human (epratuzumab) antibodies (HAHAs) were assessed at study entry and at 6, 10, and 18 weeks.

Determination of anti-Ro and anti-La autoantibodies

An indirect immunofluorescence procedure using HEp-2000 cells was employed to detect the presence and titer of ANA

Table 1

Patient demographics and baseline disease characteristics

Parameters	*n* = 15
Gender (female/male)	13/2
Age (years)	49 (33–73)
Median years post-diagnosis	2.9 (1–16)
Ocular symptoms	15 (100%)
Schirmer-I test (mm)	12 ± 12
Oral symptoms	15 (100%)
Unstimulated salivary flow (ml/minute)	0.07 ± 0.13
Moderate-to-severe fatigue	13 (87%)
Fatigue VAS (mm)	56 ± 22
Focus score ≥ 1	12 (80%)
Anti-Ro antibodies	12 (80%)
Anti-La antibodies	11 (73%)
ESR (mm/hour)	33 ± 15
IgG (mg/dl)	2,114 ± 934

ESR, erythrocyte sedimentation rate; Ig, immunoglobulin; VAS, visual analogue scale.

(Immunoconcept, Sacramento, CA, USA). Anti-Ro/SS-A and anti-La/SS-B antibodies were detected both by fluoroenzymo-immuno assay (Phadia AB, Uppsala, Sweden) and homemade double immunodiffusion. The serum levels of RF were evaluated by laser nephelometry (N Latex RF; Dade Behring, Inc., Deerfield, IL, USA). Those evaluations were performed at study entry and at 6, 10, and 18 weeks (that is, 1 day, 4 weeks, and 12 weeks post-treatment).

HAHA assay

The sponsor's [Immunomedics, Inc., Morris Plains, New Jersey, USA] HAHA test is a competitive enzyme-linked immunosorbent assay (ELISA) in which the capture reagent is epratuzumab and the probe is an anti-epratuzumab-idiotype antibody. The anti-idiotype antibody is an acceptable surrogate for what is reacted against in an immunogenic response by humans against the binding portion of epratuzumab which distinguishes the molecule from other human antibodies (that is, the framework region that has human amino acid sequences). Test results are derived from an eight-point standard curve with varying dilutions of anti-idiotype antibody in bovine serum albumin. Patient serum samples are diluted 1:2 with bovine serum albumin and assayed in triplicate. The anti-idiotype standard curve is used to determine the presence of HAHA in unknown samples. An acceptable assay is based on linear regression parameters that must be met to define a valid assay.

Statistical analysis

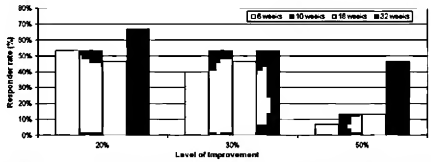
In general, discrete variables, including responder rates and AEs, were summarised using frequency counts and percentages. Percentage changes in individual efficacy parameters, B- and T-cell counts, Igs, duration of infusion times, and other continuous numerical variables were summarised using descriptive statistics. The Wilcoxon signed rank test was used to assess the statistical significance of changes in the subjective efficacy measures (VAS scores), tender points, tender joints, ESR, CRP, B cells, T cells, and Igs, compared with their baseline values. All statistical tests used a significance level of ≤0.05.

Results

Demographics and baseline disease characteristics

Sixteen patients who met the inclusion/exclusion criteria were to receive four infusions of 360 mg/m^2 epratuzumab once every 2 weeks. One patient was discontinued from the study upon receiving a partial dose of the first infusion due to a moderate acute reaction (considered as serious due to a mandatory brief overnight hospitalisation for observation). The ensuing discussion, except for safety, will focus on 15 patients who received at least one infusion of the study drug and had at least one follow-up evaluation. Their baseline characteristics are summarised in Table 1. Among the more notable medical histories, seven patients had pulmonary involvement, five had parotid enlargement, five had thyroid disease, four had neurological impairment, two had Raynaud's disease, and one had type-II cryoglobulinemia.

Figure 1



Responder rates. The overall response of a patient was determined using the four domains: dryness of the eyes (Shirmer-I test), dryness of the mouth (unstimulated whole salivary flow), fatigue (visual analogue scale), and laboratory (erythrocyte sedimentation rate and/or immunoglobulin G). A patient who achieved at least 20% improvement in at least two domains is considered a responder.

Efficacy

Fourteen patients completed the study through the 18-week (12 weeks post-treatment) evaluation period, although one among these missed the 10-week (4 weeks post-treatment) visit. The remaining patient did not receive the fourth infusion but subsequently returned for evaluation at 6 weeks. A total of 10 patients returned for the final evaluation at 32 weeks (6 months post-treatment).

General response

To assess the overall efficacy of epratuzumab in the treatment of pSS, a composite endpoint involving the Schirmer-I test, USF, fatigue VAS, and the laboratory parameters (ESR and IgG) was devised to provide a clinically meaningful definition of response, as defined in Materials and methods. Of all patients who received at least one dose of the study drug (n = 15) (Figure 1), more than half (53%) experienced a clinically meaningful response of improvement of 20% or more in two out of the four criteria at the first post-treatment evaluation at 6 weeks (24 hours after the fourth infusion). The same level of response was maintained through 10 weeks and decreased only slightly at 18 weeks (47%). At the final 32-week evaluation, 67% of the patients still showed a response that satisfied the response criteria. The corresponding results, when the response rates are calculated based on only available patients, are 53%, 62%, 50%, and 91% at 6, 10, 18, and 32 weeks, respectively.

The corresponding response rates based on improvements of at least 30% and 50% also are shown in Figure 1. Remarkably, approximately 50% of the patients also achieved the stricter responder criteria of improvement of at least 30% in two of four efficacy parameters by 10 weeks and continued to do so through 32 weeks. The corresponding response rates based on improvement levels of at least 50% were 10%–45%, depending on the visit.

Figure 2

Improvement rates in individual efficacy parameters. The improvement rate is based on achieving an improvement of at least 20% from baseline. ESR, erythrocyte sedimentation rate; IgG, immunoglobulin G; VAS, visual analogue scale.

Table 2

Subjective measures assessed by visual analogue scale (0–100 mm)

Parameters	Baseline ($n = 16$)	6 weeks ($n = 15$)	10 weeks ($n = 13$)	18 weeks ($n = 14$)	32 weeks ($n = 11$)
Fatigue	55 ± 21	46 ± 30	44 ± 28	45[a] ± 22	41[a] ± 29
Pain	49 ± 28	31 ± 32	32 ± 29	39 ± 25	34 ± 26
Patient assessment[b]	62 ± 29	38[a] ± 30	49 ± 23	48[a] ± 31	40[a] ± 28
Physician assessment[c]	56 ± 16	30[a] ± 22	38[a] ± 14	31[a] ± 20	26[a] ± 12

Results are given in mean ± standard deviation. [a]Denotes statistical significance of the observed median change-from-baseline value with $P \leq 0.05$ by Wilcoxon signed rank test. [b]Patient self-assessment of overall well-being. [c]Physician global assessment of patient's overall well-being.

Functional assessments

Schirmer-I test

More than half the patients (Figure 2) improved by at least 20% on their lacrimal function through 18 weeks, with 7/15 (47%) still showing improvement at the final visit at 32 weeks. The corresponding improvement rates based on available patients are 73%, 69%, 64%, and 64% at 6, 10, 18, and 32 weeks, respectively.

USF

Improvement by at least 20% in USF was observed in 20%–40% of the patients through 18 weeks (Figure 2), with 7/15 (47%) still showing improvement at the final visit at 32 weeks. The corresponding improvement rates based on available patients are 20%, 46%, 36%, and 64% at 6, 10, 18, and 32 weeks, respectively.

Subjective assessments

Fatigue and patient and physician global assessments

Improvement by at least 20% in fatigue was consistently observed in 40% of the patients through 32 weeks (Figure 2). The corresponding improvement rates based on available patients are 40%, 46%, 43%, and 55% at 6, 10, 18, and 32 weeks, respectively. Complete results on all subjective efficacy measures as assessed by VAS are summarised in Table 2. Statistically significant improvement from baseline was observed in fatigue and in patient and physician global assessments at several time points. There were no notable changes in pain.

Objective assessments of joint counts

Complete results on objective efficacy measures as assessed by joint counts are summarised in Table 3. In general, there were no notable changes except for a statistically significant improvement from baseline in the number of tender joints at 32 weeks.

Laboratory assessments

ESR

Improvement by at least 20% in ESR was observed in 13%–33% of the patients through 18 weeks (Figure 2), with 4/15 (27%) still showing improvement at the final visit of 32 weeks. The corresponding improvement rates based on available patients are 33%, 15%, 14%, and 36% at 6, 10, 18, and 32 weeks, respectively.

CRP

There were no statistically significant changes in CRP at any visit (Table 4).

IgG

No improvement in IgG was observed at 6 weeks, but improvement was seen subsequently in 13%, 7%, and 20% of the patients at 10, 18, and 32 weeks, respectively (Figure 2). The corresponding improvement rates based on available patients are 0%, 15%, 7%, and 27% at 6, 10, 18, and 32 weeks, respectively.

Lymphocytes and Igs

At study entry, peripheral blood lymphocyte and serum Ig levels (mean ± SD) for the 15 patients were as follows: 211 ± 111 B cells per μl, 1034 ± 426 T cells per μl, 1909 ± 669 IgG mg/dl, 291 ± 111 IgA mg/dl, and 146 ± 54 IgM mg/dl. As shown in Table 5 and Figure 3, mean B-cell levels decreased by 54% at 6 weeks, which persisted at subsequent evaluations, with no evidence of onset of recovery by the final evaluation at 32 weeks (6 months post-treatment). In contrast, there were no consistent patterns of decreases/increases either in

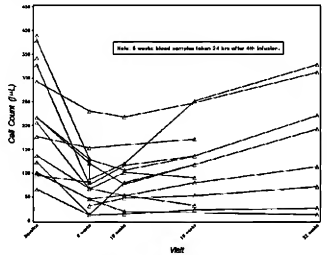
Table 3

Number of tender points and tender joints

Parameters	Baseline ($n = 16$)	6 weeks ($n = 15$)	10 weeks ($n = 13$)	18 weeks ($n = 14$)	32 weeks ($n = 11$)
Tender points	4.1 ± 5.6	2.0 ± 3.1	2.1 ± 3.4	1.6 ± 2.9	2.4 ± 3.3
Tender joints	4.0 ± 7.5	1.0 ± 1.8	1.1 ± 1.6	1.2 ± 1.9	0.3[a] ± 0.5

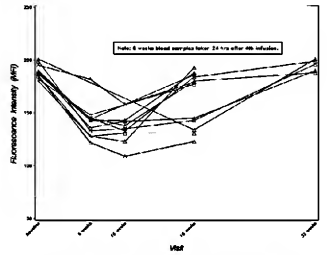
Results are given as mean ± standard deviation. [a]Denotes statistical significance of the observed median change-from-baseline value with $P \leq 0.05$ by Wilcoxon signed rank test. Almost all patients had no swollen joints at baseline or subsequent time points.

Figure 3



Peripheral B-cell counts.

Figure 4



CD22 expression on B cells as measured by mean fluorescence intensity (MFI).

the T-cell levels or in the available serum levels of IgG, IgA, and IgM after treatment.

At study entry, all of the patients with available measurements had a CD22 median fluorescence intensity above the normal range (from median 88 to 201). This is consistent with the finding that patients with Sjögren's syndrome have an over-expression of CD22. Twenty-four hours after treatment with epratuzumab at 6 weeks, all but one patient exhibited a decreased CD22 fluorescence intensity below the normal range (Figure 4). At week 18, five patients remained CD22 downregulated, but the others returned to a fluorescence intensity as high as at entry. At the final evaluation, all the patients recovered to the same increased CD22 expression compared with normal, as observed in patients with untreated pSS.

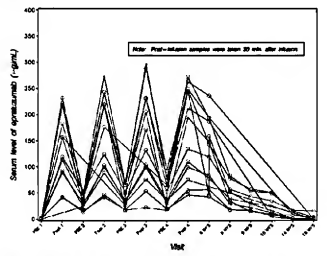
Autoantibodies

There were no changes in the autoantibodies, anti-Ro and anti-La, in any patients with at least one post-treatment measurement; specifically, in subsequent visits, no patient developed new autoantibodies that were not also detected at study entry. Almost all patients (13/15 with at least one post-treatment measurement) had measurable ANA titers (1:80 to 1:10,000) at study entry. At subsequent evaluations, including the 32-week (6 months post-treatment) visit, eight patients exhibited at least twice their baseline ANA titers at one or more evaluations, whereas a similar number (seven patients) had not more than half their baseline titers at one or more evaluations, with two of the aforementioned patients having both increases and decreases from their baseline titers at different evaluation time points.

Study drug administration and reactions

A total of 16 patients were exposed to the study drug. One patient experienced a moderate-severe acute reaction (flushing, dyspnea, nausea, vomiting, nasal mucosa swelling, and glottis pressure) during the first infusion and was discontinued from the study. Of the remaining 15 patients, 14 (93%) completed all four infusions of 360 mg/m^2 of epratuzumab and one prematurely terminated the third infusion after experiencing a moderate grade-3 acute infusion reaction (with a loss of consciousness for several seconds) that subsided within 1 hour (the fourth infusion was not administered to this patient). Overall, the infusions were administered in a median infusion time

Figure 5



Serum levels of epratuzumab, as measured by enzyme-linked immunosorbent assay.

Table 4

Post-treatment changes in CRP, ESR, and Igs

Parameters	Post-treatment change from baseline (mean ± standard deviation)			
	6 weeks	10 weeks	18 weeks	32 weeks
	n = 13	*n* = 13	*n* = 12	*n* = 10
CRP	0.42 ± 1.39	-0.02 ± 0.33	0.19 ± 0.60	0.03 ± 0.22
ESR	1.3 ± 15.0	1.5 ± 11.0	-0.9 ± 7.7	-0.3 ± 11.6
	n = 15	*n* = 13	*n* = 13	*n* = 9
IgG	29.7 ± 249.6	-31 ± 379.6	7.1 ± 313.5	-120.8 ± 363.3
IgA	1.1 ± 24.4	7.8 ± 46.9	-1.2 ± 59	-3.8 ± 62.4
IgM	-14.9 ± 25.3	-9.6 ± 28.3	-15.0 ± 32.8	-2.3 ± 34.8

None of the changes from baseline in the above parameters was statistically significant. CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; Ig, immunoglobulin.

of 45 minutes (20–150 minutes) and were generally well-tolerated, with four transient AEs (headache, lower limb paresthesia, and two cases of acute infusion reaction) that resolved quickly.

Safety

Safety assessments focus on all 16 patients who were exposed to study medication.

Adverse events

During or after treatment, a total of 10 patients reported AEs. Four reported having a serious AE (drug-related: acute infusion reaction as noted above; non-drug-related: dental abscess, transient ischemic attack with secondary seizure, and osteoporotic fracture), and three patients had a non-serious AE considered drug-related (headache, paresthesia, and acute infusion reaction as noted above) that resolved quickly. The remaining AEs considered unrelated to study medication included fever, palpitation, bone pain, sinusitis, carpal tunnel syndrome, diarrhea, and dyspepsia. The two cases of infection reported above (sinusitis and dental abscess treated with intravenous antibiotics) resolved subsequently without any sequelae.

Safety laboratories

Standard safety laboratories showed no consistent pattern of change from baseline, and infrequent post-treatment increases in National Cancer Institute Common Toxicity Criteria (CTC) (version 3.0) toxicity grades for these laboratories were all limited to changes of at most one grade level, except for one patient with lymphopenia that increased from CTC grade 0 to 2.

Immunogenicity

HAHA analyses showed three patients with elevated values of HAHA: 116 ng/ml at 32 weeks, 120 ng/ml at 18 weeks, and 130 ng/ml at 18 weeks. These isolated cases of low-level positive HAHA are of uncertain clinical significance because they were not associated with specific clinical signs and symptoms or other apparent toxicities.

Pharmacokinetics

Serum samples for analysis of pharmacokinetics by ELISA were collected pre- and post-infusion as well as at 6 weeks (24 hours after fourth infusion) and 8, 9, 10, 14, and 18 weeks. Epratuzumab serum levels were detectable above the 0.5 μg/ml assay limit in all 13 available samples at 6 weeks, in 10/11 samples evaluated at 10 weeks, in 6/10 samples evaluated at 14 weeks, and in 6/14 samples evaluated at 18 weeks, with median values of 143 μg/ml (range, 43–236) at 6 weeks, 14 μg/ml (4–51) at 10 weeks, 11 μg/ml (1–17) at 14 weeks, and 3.9 μg/ml (1–16) at 18 weeks (Figure 5). Non-compartmental pharmacokinetic analysis indicated a serum half-life ($t_{1/2}$) after the fourth infusion of 15 ± 8 days.

Discussion

In this phase I/II open-label study, selective immunomodulation of B cells led to improvement of objective and subjective parameters of disease activity in patients with pSS. In the absence of validated disease activity criteria for pSS, we developed a disease activity score based on the most frequent signs and symptoms of the disease. These included four domains: dryness of the eyes (Schirmer-I test), dryness of the mouth (USF), fatigue (VAS), and laboratory parameters of ESR and/or IgG. Based on this activity score, we observed that more than half (53%) of the patients achieved at least a 20% improvement in at least two domains 24 hours after the fourth infusion at 6 weeks, with the corresponding response rates of

Table 5

Post-treatment changes in lymphocytes

	Post-treatment percentage change from baseline (mean ± SD)			
Parameter	6 weeks	10 weeks	18 weeks	32 weeks
Lymphocytes	$n = 14$	$n = 12$	$n = 11$	$n = 7$
B cells	-54%* ± 25%	-45%* ± 47%	-39%* ± 23%	-31%* ± 33%
T cells	2% ± 36%	-6% ± 34%	1% ± 13%	-5% ± 19%

*Denotes statistical significance of the observed median change-from-baseline value with $P \leq 0.05$ by Wilcoxon signed rank test. None of the changes from baseline in T cells was statistically significant. SD, standard deviation.

53%, 47%, and 67% at 10, 18, and 32 weeks, respectively. Most improvements occurred in the Schirmer-I test, USF, and fatigue VAS. There appear to be only a few significant changes in ESR or IgG that impacted on the efficacy outcomes. Approximately 40%–50% responded at the improvement level of at least 30%, whereas 10%–45% responded at the 50% improvement level for 10–32 weeks. Interestingly, the number of responders (at 20%, 30%, or 50% improvement levels) was higher 6 months after the treatment administration than earlier. Such results might indicate recovery or regeneration of glandular tissue, suggesting the need for pre- and post-therapy biopsies of minor salivary glands. Additional findings in terms of statistically significant improvement from baseline in fatigue, and in patient and physician global assessments at several time points, also serve to reinforce the positive results based on the composite efficacy endpoint.

B-cell targeting therapies are promising in the treatment of autoimmune diseases, including rheumatoid arthritis [27] and SLE [23]. Also, two studies and some case reports evaluated the anti-CD20 monoclonal antibody, rituximab, in pSS [10-13]. Pijpe *et al.* reported observing a significant improvement in subjective and objective measures, including subjective reports of dryness, fatigue, and salivary flow, mainly in patients with early-onset disease and in only a few with pSS-associated MALT (mucosa-associated lymphoid tissue) lymphoma [11]. Immunologic analysis showed rapid decrease in peripheral B cells but no change in IgG levels. However, four of 14 patients developed human anti-chimeric antibodies (HACAs) and three of them developed a clinical picture compatible with serum sickness [11]. In a retrospective study of short-term efficacy of rituximab in autoimmune diseases, serum-sickness-like diseases occurred in a patient with pSS and in two patients with SLE [12]. This is supported by another French, open-label study using low-dose rituximab which reported improvement in subjective parameters of dryness and which also showed that two of 16 patients developed a clinical picture of serum sickness [13]. The most striking finding in those studies is the observation of HACA-associated serum sickness, which may be of major clinical concern in future trials. Accordingly, fully humanised anti-CD20 monoclonal antibodies are under evaluation in autoimmune diseases, in addition to NHL [28-30]. Nevertheless, all of these CD20 antibodies appear to markedly deplete circulating B cells in treated patients.

Although depleting B cells is interesting in the treatment of autoimmune diseases, a novel and rational approach is modulating their function. Initial data have shown that epratuzumab is effective and safe in the treatment of SLE [23]. This treatment was associated with a modest depletion of B cells (34%–41%) within 18 weeks, as we observed also in the present study (39%–54% within 18 weeks) and as was also found in patients with NHL treated with epratuzumab [22]. However, it might also function by signalling through the inhibitory CD22 molecule, causing down-modulation of BCR signalling, as suggested in recent laboratory studies comparing epratuzumab with rituximab [21]. B-cell homeostasis is disturbed in pSS with diminished frequencies and absolute numbers of peripheral $CD27^+$ memory B cells [4-7]. In addition, we report here, for the first time, that patients with pSS have a CD22 over-expression in their peripheral B cells, which was downregulated by epratuzumab for at least 12 weeks after the therapy.

In addition to assessing any evidence of efficacy, the objective of this open-label phase I/II study was to evaluate the safety of epratuzumab in patients with active pSS. Three patients showed moderately elevated levels of HAHA, but without any specific clinical symptoms or apparent toxicity that could be associated with the elevations. As compared with patients with lymphoma, those suffering from autoimmune diseases have been reported to present a higher rate of antibodies to chimeric rituximab, but usually not related with clinical manifestations [27,31]. These discrepancies may be explained in part by the high B-cell activity in pSS and the lack of concomitant immunosuppressive therapy.

Conclusion

This initial experience in patients with active pSS demonstrated that four doses of 360 mg/m² epratuzumab immunotherapy appears to be safe and well-tolerated when infused within 45 minutes, with clinically significant responses observed in approximately half the patients for at least 18 weeks in the presence of modestly decreased (39%–54%)

circulating B-cell levels, and with evidence of minimal immunogenicity, as measured by HAHA. We conclude that epratuzumab may be a promising therapy in patients with active pSS and that a multicentre, randomised, double-blinded, controlled study to confirm the beneficial effects of anti-CD22 therapy is indicated.

Competing interests

SDS and GRB declare research funding for this study provided by Immunomedics, Inc. SDS has acted as a research consultant for Genentech, Inc. NKWT, WAW, and DMG have employment and financial interests (stock) in Immunomedics, Inc., whichowns the antibody tested in this paper. OP and LT declare no competing interests.

Authors' contributions

All authors contributed to data interpretation and the final manuscript. SDS and GRB were the principal investigators and were responsible for all aspects of the study, including patient selection and performing patient-related study procedures. SDS, GRB, DMG, and WAW designed the clinical trial protocol, and NKWT was responsible for data management and statistical analysis. All authors read and approved the final manuscript.

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Genentech
IN BUSINESS FOR LIFE

Friday, Mar 17, 2006

Genentech Provides Updates at Annual Investment Meeting

South San Francisco, Calif. -- March 17, 2006 -- Genentech, Inc. (NYSE: DNA) today provided an overview of its business goals for 2006 and beyond, including an update of its Horizon 2010 strategic plan, at its investment community meeting in New York. In addition, the company provided investors with an overview of recent developments, including highlights from its research, development, commercial, and manufacturing efforts.

"Our success over the past several years has transformed our business, leading to additional opportunities for growth," said Chairman and Chief Executive Officer Arthur D. Levinson, Ph. D. "We remain focused on understanding the basic scientific mechanisms of disease, so that we are better able to select the right targets quickly, develop those targets in the clinic, and deliver novel therapies that could change the course of some of the deadliest diseases."

Horizon 2010 Goals
The company provided an update of its long-term business objectives. The company's revised Horizon 2010 goals include:

- To bring at least 20 new molecules into clinical development.
- To bring at least 15 major new products or indications onto the market.
- To become the number one U.S. oncology company in sales.
- To achieve an average compound annual non-GAAP earnings per share[1] growth rate of 25 percent.
- To achieve cumulative free cash flow[2] of $12 billion.

Other Business Updates
The company made the following additional announcements today:

- The company expects approximately 40 to 50 percent growth in non-GAAP earnings per share for the full year 2006.[3]
- The company announced that the anti-CD20 humanized monoclonal antibody (ocrelizumab) Phase I/II ACTION study in rheumatoid arthritis met its primary endpoint of safety in all doses studied and also met its secondary endpoint of clinical activity at all dose levels studied. The most common side effects in ocrelizumab-treated patients included mild to moderate adverse events of nausea, chills or headache around the first infusion. The serious adverse events observed in patients treated with ocrelizumab were similar to those seen in patients receiving placebo. There were no serious infusion related events in patients treated with ocrelizumab. The rates of infection-related adverse events were similar

between placebo and active groups. Further analyses of the data are ongoing and will be submitted for presentation at a future medical meeting.

- The company announced its decision to acquire land in Hillsboro, Oregon for the construction and development of a biotherapeutic fill/finish manufacturing facility, which is expected to be licensed and operational in 2010.
- In February 2006, Genentech purchased from Biogen Idec the NICO clinical manufacturing facility in Oceanside, California, which will add approximately 5,500 liters of capacity to be used for clinical manufacturing of new molecular entities.
- Genentech also announced that the U.S. Food and Drug Administration approved in January 2006 the production of Xolair® (Omalizumab) bulk drug substance at Novartis' production facility in Huningue, France.

Webcast

Genentech will be offering an archived webcast of the investment community meeting on its website at http://www.gene.com. The webcast will be archived and available for replay until 8:00 p.m. Eastern Time on March 31, 2006.

About Genentech

Genentech is a leading biotechnology company that discovers, develops, manufactures and commercializes biotherapeutics for significant unmet medical needs. A considerable number of the currently approved biotechnology products originated from or are based on Genentech science. Genentech manufactures and commercializes multiple biotechnology products and licenses several additional products to other companies. The company has headquarters in South San Francisco, California and is listed on the New York Stock Exchange under the symbol DNA. For additional information about the company, please visit http://www.gene.com.

Notes

[1] Non-GAAP earnings per share estimates for this period exclude the after-tax effects of recurring charges related to the 1999 Roche redemption of our common stock, ongoing special charges related to the City of Hope litigation, stock compensation expense,and any potential special charges related to existing or future litigation or its resolution, or changes in accounting principles, all of which could be significant. [2] Free cash flow, a non-GAAP measure, will be computed by Genentech based on operating cash flow less gross capital expenditures. Operating cash flow is derived from the "net cash provided by operating activities" line in the cash flow statement and excludes the after-tax effects of non-operational items related to our investment portfolio, asset dispositions, litigation costs, debt service costs, and any other potential non-operational items which could affect this line, any of which could be significant. [3] Genentech's forecasted 2006 non-GAAP earnings per share exclude the after tax-effects of recurring charges related to the 1999 Roche redemption of our

common stock estimated to be $105 million on a pretax basis in 2006, special charges related to the City of Hope litigation estimated to be $54 million on a pretax basis in 2006,stock compensation expense associated with Genentech's adoption of SFAS No. 123R on January 1,2006,expected to be in the range of $0.15 to $0.17 per share for 2006 and any other potential special items related to existing or future litigation or its resolution, or changes in accounting principles, all of which could be significant.

This press release contains forward-looking statements regarding growth in non-GAAP earnings per share and cumulative free cash flow; adding 20 new molecules into clinical development and 15 major new products or indications onto the market by 2010; becoming the number one U.S. oncology company in sales; licensure, development and operation of manufacturing facilities; and charges related to the 1999 Roche redemption of Genentech's stock, the City of Hope litigation and stock compensation. Such statements are predictions and involve risks and uncertainties and actual results may differ materially. Among other things, adding molecules into clinical development, adding products or indications into the market, and the licensure, development and operation of manufacturing facilities could be affected by a number of factors, including unexpected safety, efficacy or manufacturing issues,additional time requirements for data analysis, and FDA actions or delays including failure to obtain FDA approval; becoming a leader in oncology sales could be affected by all of the foregoing and by a number of other factors, including competition,pricing, reimbursement,the ability to supply product, product withdrawals and new product approvals and launches; charges related to the 1999 Roche redemption of Genentech's stock, the City of Hope litigation and stock compensation could be affected by a number of factors, including a re-valuation of certain intangible assets, greater than expected litigation-related costs, the number of options granted to employees, Genentech's stock price and certain valuation assumptions concerning Genentech stock; and growth in non-GAAP EPS and cumulative free cash flow, could be affected by all of the foregoing and by a number of other factors, including achieving sales revenue consistent with internal forecasts, unanticipated expenses such as litigation or legal settlement expenses or equity securities writedowns, costs of sales, R&D expenses, fluctuations in contract revenues and royalties, and fluctuations in tax and interest rates. Please also refer to Genentech's periodic reports filed with the Securities and Exchange Commission. Genentech disclaims, and does not undertake, any obligation to update or revise any forward-looking statements in this press release.

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Evaluating Antibodies for Their Capacity to Induce Cell-mediated Lysis of Malignant B Cells[1]

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ABSTRACT

Promising results from clinical trials have led to renewed interest in effector mechanisms operating in antibody-based therapy of leukemia and lymphoma. We tested a panel of B-cell antibodies from the Sixth Human Leukocyte Differentiation Antigen workshop for their capacity to mediate antibody-dependent cellular cytotoxicity, often considered to be one of the most potent effector mechanisms *in vivo*. As effector cells, mononuclear cells and polymorphonuclear (PMN) cells from healthy donors were compared with FcγRI (CD64)-expressing PMN cells from patients receiving granulocyte colony-stimulating factor (G-CSF) treatment. Of the 29 IgG workshop antibodies binding most strongly to the tested malignant human B-cell lines, only 3 consistently induced target cell lysis. These three antibodies were determined to be HLA DR reactive. Experiments with a panel of HLA class II antibodies showed the involvement of individual Fcγ receptors on effector cells to be strongly dependent on the antibody isotype. We then compared killing mediated by chimeric IgG1 antibodies with that from FcγRI-directed bispecific antibodies, targeting classical HLA class II, or the Lym-1 and Lym-2 antigens. The latter two are variant forms of HLA class II, which are highly expressed on the surface of malignant B cells but which are found only at low levels in normal cells. With blood from G-CSF-treated donors, bispecific antibodies showed enhanced killing compared to their chimeric IgG1 derivatives, because they were more effective in recruiting FcγRI-expressing PMN cells. G-CSF- and FcγRI-directed bispecific antibodies to HLA class II, therefore, seem to be an attractive combination for lymphoma therapy.

INTRODUCTION

Malignant lymphomas are the most common neoplasm of young adults, with increasing mortality over the last decades (1). In the Western world, most cases are of B-cell origin, and, although chemo- and radiotherapy have proven to be effective treatments, the majority of patients with disseminated low-grade lymphoma or relapses of high-grade lymphoma will ultimately die from their disease. The application of MoAbs[3] has the potential to become another therapeutic option (2). Hematological malignancies seem to be particularly promising targets for antibody therapy, because antibodies to well-defined and rather specific surface molecules are available, therapeutic antibodies usually reach their targets, and induction of human antimouse or antichimeric antibody is less pronounced than in patients with solid tumors (3). Clinical trials with customized antibodies to patients' tumor idiotype were the first to show encouraging results in lymphoma patients (4), and IDEC-C2B8 [a chimeric CD20 antibody (5)] was the first MoAb to be approved by the United States Food and Drug Administration for treatment in oncology.

MoAbs mediate their antitumor effects either by directly acting on tumor cells (*e.g.*, by blocking growth factors, inhibiting cell proliferation, or inducing programmed cell death or dormancy) or by recruiting immune effector mechanisms such as cell- or complement-dependent cytotoxicity. Studies with isotype switch variants showed a positive correlation between the capacity to induce ADCC *in vitro* and therapeutic efficacy *in vivo*, suggesting that ADCC can be an important mechanism of antibody action *in vivo* (6). Neutrophils, the most abundant Fc receptor-expressing effector cells, showed cytolytic activity against a broad spectrum of tumor cells *in vitro* (7) and were critically involved in the rejection of cytokine-transfected tumor cells *in vivo* (8). *In vitro*, we found cell-mediated target cell lysis by neutrophils to be a major effector mechanism for HER-2/neu-directed MoAbs (9). The contribution of neutrophils can be further enhanced by clinical application of hematopoietic growth factors, such as G-CSF or granulocyte-macrophage CSF, which dramatically raise neutrophil numbers *in vivo* and at the same time stimulate important functions, such as phagocytosis, release of oxygen radicals, and ADCC (10).

Cell-mediated effects of MoAbs require interaction between the Fc region of antibodies with activating Fc receptors on immune effector cells (11). Depending on their specificity for the heavy chains of IgA, IgE, or IgG, Fc receptors are grouped as Fcα, Fcε, or Fcγ receptors, respectively (12, 13). The majority of Fc receptors consist of ligand-specific α chains, which associate with shared molecules for signaling (14, 15). Neutrophils constitutively express the myeloid receptor for IgA (FcαRI, CD89) and two low-affinity IgG receptors, FcγIIa (CD32) and FcγIIIb (CD16; Ref. 16). IFN-γ (17) or G-CSF (18) induces neutrophils to additionally express the high-affinity IgG receptor (FcγRI, CD64). Tumor-cytolytic activity on neutrophils has been established for FcγRI, FcγRII and, more recently, for FcαRI (19) but not for FcγRIIIb, which is glycosylphosphatidylinositol-linked on PMN cells. Monocytes/macrophages mediate tumor cell killing via molecules belonging to all three Fcγ receptor classes, whereas NK cells express only the cytotoxically active FcγRIIIa (11).

In a previous study, comparing the capacity of B cell-directed antibodies to induce ADCC of malignant cells, we observed an unexpected antigen restriction whereby PMN cells induced high levels of target cell lysis with antibodies to HLA class II but not with antibodies to classical B-cell antigens, such as CD19, CD20, CD21, CD37, or CD38 (20). Here, we report on our results with an extended panel of B-cell antibodies from the Sixth Human Leukocyte Differentiation Antigen workshop and with engineered antibody derivatives. The results extend our understanding of HLA class II-directed reagents and their ability to recruit effector cells and underscore the potential of bispecific antibodies as therapeutic reagents in the treatment of lymphoma.

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[3] The abbreviations used are: MoAb, monoclonal antibody; ADCC, antibody-dependent cellular cytotoxicity; CSF, colony-stimulating factor; G-CSF, granulocyte CSF; PMN, polymorphonuclear; MNC, mononuclear cell; *o*-PDM, *o*-phenlendimaleimide; RFI, relative fluorescence intensity; NK, natural killer.

PATIENTS AND METHODS

Blood Donors. Experiments reported here were approved by the Ethical Committee of the University of Erlangen-Nürnberg, in accordance with the Declaration of Helsinki. After informed consent was obtained, peripheral blood was drawn from healthy volunteers or from patients receiving G-CSF therapy. Patients were treated with rh-met-G-CSF (Neupogen; 3–5 μg/kg of body weight) from Hoffmann La-Roche (Basel, Switzerland), based on clinical indications. In G-CSF treated patients, FcγRI expression on PMN cells was significantly ($P < 0.001$) higher than in healthy donors, as reported (18).

Isolation of Mononuclear and Neutrophil Effector Cells. Mononuclear and neutrophil effector cells were isolated as described (20). Briefly, 10–20 ml of citrate anticoagulated blood was layered over a discontinuous Percoll (Seromed, Berlin, Germany) gradient. After centrifugation, neutrophils were collected at the interphase between the two Percoll layers, and MNCs were collected from the Percoll/plasma interphase. Remaining erythrocytes were removed by hypotonic lysis. Purity of neutrophils was determined by cytospin preparations and exceeded 95%, with few contaminating eosinophils and <1% MNCs. Viability was tested by trypan blue exclusion and was higher than 95%.

Cell Lines. Malignant human B-cell lines REH (O-acute lymphoblastic leukemia), RAJI (Burkitt's lymphoma), ARH-77 (mature B cells), HUT-78 (T-acute lymphoblastic leukemia) and L cells (mouse fibroblasts) were obtained from the American Type Culture Collection (Manassas, VA). RM-1 (an EBV-transformed B-cell line) was from Dr. G. Bonnard (Bethesda, MD, Ref, 21), and JK-6 (plasmocytoma) was established at our institution by Dr. R. Burger (22). All cells were kept in RF10$^+$ medium consisting of RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, 100 units/ml streptomycin, and 4 mmol/liter L-glutamine (all from Life Technologies).

Generation of HLA Class II Transfectants. Vectors containing DRA *0101 (DRα 120; ATCC 57392) and DRB1 *0101 (45.1 DRβ 008; ATCC 57081) were obtained from the American Type Culture Collection. Sense primers CCC-AAG-CTT-CGA-GCT-CTA-CTG-ACT (DRα) and CCC-AAG-CTT-GGT-CCT-GTC-CTG-TTC (DRβ), and antisense primers CCC-TCT-AGA-AAG-TTT-CTT-CAG-TGA (DRα) and CCC-TCT-AGA-GAA-GGT-TCT-TCC-TTG (DRβ) were used to amplify DRα and DRβ, respectively, cDNA from the plasmids and to generate singular *Hin*dIII and *Xba*I restriction sites at 5' and 3' ends. PCR products were cloned into pGEM-T-vector (Promega, Madison, WI) and sequenced by the dye terminator method on an ABI Prism automatic sequencer (Applied Biosystems, Foster City, CA). For eukaryotic expression, inserts were ligated via their unique restriction sites into pRc/CMV (Invitrogen, NV Leek, the Netherlands). Transfection of 10^7 L-cells was carried out by simultaneous electroporation of 10 μg each of the DRα and DRβ expression vectors at 250 V and 960 μF (Bio-Rad, Richmond, CA). After 24 h, G418 (Life Technologies) was added to the culture medium for selection of stable transfectants. High-expressing cells were obtained by fluorescence activated cell sorting on an EPICS ELITE ESP (Coulter, Hialeah, FL) after staining with HLA class II antibody F3.3 and FITC-labeled goat antimouse F(ab')$_2$.

MoAbs and Antibody Constructs. B-cell panel antibodies were from the Sixth Human Leukocyte Differentiation Antigen workshop (Table 1). Hybridomas of negative control antibody 3.6.2 (mIgG2a) and L243 (HLA DR, mIgG2a) were from the American Type Culture Collection. HLA class II antibody F3.3 (mIgG1) and invariant chain (Ii, CD74) antibody AT14/15 (mIgG1) were produced at Tenovus Research Laboratory (University of Southampton, Southampton, United Kingdom). HLA DR (B8.11.2, mIgG2b)-, DP (B7.21, mIgG2a)-, and DQ (SPV L3, mIgG2a)-specific antibodies were kindly provided by Dr. R. Bontrop (Biomedical Primate Research Center, Rijswijk, the Netherlands). Murine Lym-1 (mIgG2a) and Lym-2 (mIgG1) and their mouse/human chimeric IgG1 constructs were produced as described (23, 24).

Fc receptor antibodies 22 and 197 (mIgG1 and IgG2a, respectively), both to FcγRI; IV.3 (mIgG2b) to FcγRII; and 3G8 (mIgG1) to FcγIII, as well as F(ab') and F(ab')$_2$ fragments of IV.3 and 3G8, respectively, were kindly provided by Medarex (Annandale, NJ).

Bispecific antibodies [FcγRI × HLA class II], [FcγRI × Lym-1], and [FcγRI × Lym-2] were produced by chemically cross-linking F(ab') fragments of trigger molecule MoAb 22 (FcγRI; CD64) with F(ab') fragments of target antigen antibodies F3.3, Lym-1, or Lym-2, as described (25). Briefly, F(ab')$_2$ fragments were produced by limited digestion with pepsin and were then reduced with mercaptoethanol amine to provide F(ab') with free hinge-region sulfur hydroxyl (SH) groups. The SH groups on one of the Fab'-γ (SH) partners were then fully derivatized with excess *o*-PDM to provide free maleimide groups. Finally, the F(ab')-*o*-PDM and F(ab')-SH were combined at a ratio of 1:1 to generate heterodimeric F(ab')-*o*-PDM-F(ab') constructs. After purification by size exclusion chromatography and characterization by high-performance liquid chromatography, samples were sterilized by filtration and stored at 4°C. All bispecific antibodies showed binding to effector and target cells as expected from their parental antibodies.

Chimeric Fab(Fc)$_2$ constructs of HLA class II antibody F3.3, consisting of F(ab') fragments from the mouse antibody chemically conjugated to two human Fc fragments, were prepared as reported (26). Briefly, F(ab')-*o*-PDMs of F3.3 were produced as described above. To prepare Fcγ, normal human IgG was digested with papain, and the resulting Fcγ fragments were separated and purified. Following reduction of Fcγ fragments, fragments were incubated with F(ab')-*o*-PDM to yield Fab(Fc)$_2$ constructs with mainly human IgG1 Fc fragments.

Immunofluorescence Analysis. During incubation of effector cells with MoAbs, polyeloinal human IgG (4 mg/ml) was added to inhibit nonspecific binding to FcγRI. FITC-labeled F(ab')$_2$ fragments of goat antimouse or anti-human MoAbs were used for staining. Cells were analyzed on an EPICS PROFILE flow cytometer (Coulter). For each cell population, RFI was calculated as the ratio of mean linear fluorescence intensity of relevant to irrelevant, isotype-matched antibodies.

ADCC Assays. ADCC assays were performed as described (20). Briefly, target cells were labeled with 200 μCi of ^{51}Cr for 2 h. Effector cells, sensitizing antibodies, and RF10$^+$ were added into round-bottomed microtiter plates. In some experiments, Fcγ receptor-blocking antibodies were used at a final concentration of 10 μg/ml. Assays were started by adding the target cell suspension, giving an E:T cell ratio of 40:1. For whole blood assays, 50 μl of whole blood were added instead of isolated effector cells. After 3 h at 37°C, assays were stopped by centrifugation, and ^{51}Cr release from triplicates was measured in cpm. The percentage of cellular cytotoxicity was calculated using the formula:

Table 1 *Binding of B-cell panel antibodies to B-cell lines of different maturation levels*

One hundred two antibodies from the Sixth Human Leukocyte Differentiation Antigen workshop were tested for binding to four different human B-cell lines. Relative fluorescence intensities, antibody isotypes, and antibody specificity, as determined by the workshop, are listed for the 29 most strongly binding antibodies.

			Cell line			
Antibody	Isotype	Antigen	REH	RAJI	RM-1	JK-6
B001	IgG1	CD45RA	13.9	45.6	44.2	3.4
B005	IgG1	CD138	0.9	0.83	21.4	51.0
B006	IgG1	Unknown	0.9	1.3	29.8	10.7
B019	IgG1	Unknown	13.0	47.7	56.8	1.2
B022	IgG1	CD19	56.6	18.0	4.0	0.9
B023	IgG1	CD22	6.1	21.7	2.0	1.0
B025	IgG1	CD37	1.6	37.0	15.9	0.9
B026	IgG2a	Unknown	1.3	1.7	2.2	19.0
B028	IgG1	CD24	37.7	2.7	2.5	1.2
B033	IgG1	Unknown	1.0	10.3	6.7	4.6
B035	IgG2a	CD55	1.6	1.6	3.4	6.4
B042	IgG1	Unknown	3.0	2.0	23.7	29.3
B044	IgG1	Unknown	11.6	42.1	1.1	50.3
B046	IgG1	CD9	62.4	1.0	0.7	1.3
B048	IgG1	CD79	1.6	7.5	2.1	4.9
B049	IgG1	Unknown	1.1	1.0	1.2	8.3
B054	IgG1	Unknown	9.3	1.3	5.4	1.7
B065	IgG1	Class II	2.0	1.9	30.0	2.6
B066	IgG3	κ	2.0	1.6	0.8	8.0
B067	IgG1	CD39	2.3	2.6	28.4	1.1
B068	IgG2a	Class II	3.0	2.6	12.9	0.5
B069	IgG3	DR	35.8	87.3	67.9	1.9
B070	IgG3	DR	37.2	87.3	56.8	1.1
B071	IgG3	DR	28.9	13.0	6.5	0.8
B076	IgG2a	IgM	2.3	44.2	7.6	0.8
B077	IgG1	CD19	51.3	32.0	6.3	0.6
B095	IgG1	CD21	4.6	25.8	2.1	1.5
B096	IgG1	CD10	52.1	19.2	2.5	1.3
B099	IgG1	CD21	1.4	20.8	1.1	0.8

$$\% \text{ specific lysis} = \frac{\text{Experimental cpm} - \text{basal cpm}}{\text{Maximal cpm} - \text{basal cpm}} \times 100$$

with maximal ^{51}Cr release determined by adding perchloric acid (3% final concentration) to target cells and basal release measured in the absence of sensitizing antibodies and effector cells. Only very low levels of antibody-mediated, noncellular cytotoxicity (without effector cells) were observed under these assay conditions (<5% specific lysis). Antibody independent killing was seen in whole blood assays and with mononuclear effector cells, but not with PMN cells. For analysis of effects induced by Fc receptor antibodies, percentage of inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\% \text{ lysis without} - \% \text{ lysis with FcγR antibody}}{\% \text{ lysis without FcγR antibody}} \times 100$$

Statistical Analysis. Data are reported as mean ± SE from an indicated number of experiments with different blood donors. Differences between groups were analyzed by unpaired (or, when appropriate, paired) Student's *t* test. Levels of significance are indicated.

RESULTS

ADCC Activity of the B-Cell Panel Antibodies from the Sixth Human Leukocyte Differentiation Antigen Workshop. A total of 102 B-cell antibodies from the Sixth Human Leukocyte Differentiation Antigen workshop were tested for binding to four human B-cell lines representing different stages of maturation: REH (O-acute lymphoblastic leukemia), RAJI (Burkitt's lymphoma), RM-1 (mature B cells), and JK-6 (plasmocytoma). Binding intensities of the 29 most strongly binding IgG antibodies, their isotypes, and their antigen specificities, as determined by the B-cell workshop, are summarized in Table 1. These antibodies were then analyzed for their capacity to mediate ADCC against respective target cell lines. As effector cells, we compared PMN cells (data not shown) and MNCs from healthy donors with PMN cells from patients during G-CSF treatment (Fig. 1). Antibodies B069, B070, and B071 were found to give the highest levels of killing on the broadest spectrum of target cells. These three antibodies, as well as B065 and B068, were determined by the workshop to be HLA class II reactive. B065 and B068, however, bound only weakly to most of the tested target cells (Table 1) and did not mediate ADCC. MNCs, additionally, mediated ADCC against RAJI cells in the presence of antibodies B025 and B099, which were clustered as CD37 and CD21, respectively. Plasmocytoma cell line JK-6 is HLA class II negative and was not lysed with any of the tested panel antibodies. However, JK-6 cells, coated with the hapten nitroiodophenyl, were killed by PMN cells and MNCs in the presence of nitroiodophenyl-directed antibodies, excluding the possibility that they were resistant to the lytic mechanisms of ADCC (data not shown). Interestingly, PMN cells were cytotoxic only in the presenee of HLA class II antibodies, as previously reported (20).

Isotype Dependency of MNC- and PMN Cell-mediated ADCC. Antibody isotype has been reported to be a critical factor for effective ADCC (6). Therefore, we analyzed HLA class II antibodies of different isotypes for their capacity to mediate ADCC with PMN cells and mononuclear effector cells. As expected from the isotype specificities of FcγRIIIa on NK cells (16), MNCs were most effective with antibodies of human IgG1 or murine IgG3 isotypes. PMN cells from healthy donors or from G-CSF-treated patients induced significant target cell killing with antibodies of human IgG1, as well as with all

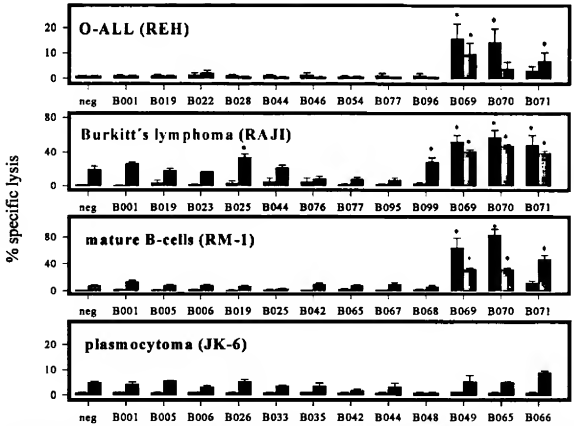

Fig. 1. ADCC capacity of B-cell panel antibodies against malignant B-cell lines. The 12 IgG antibodies with the highest staining intensities on four human B-cell lines (see Table 1) were tested at 2 μg/ml in ADCC against malignant B cells of different maturation levels. As effector cells, G-CSF-primed PMN cells (■) or MNCs (□) were used at an E:T ratio of 40:1. Significant ADCC (•) was observed most consistently with antibodies B069, B070, and B071, which were directed against HLA DR and, therefore, stained negative on plasmocytoma cell line JK-6. Results from four experiments with different donors are presented as mean ± SE of the percentage of specific lysis.

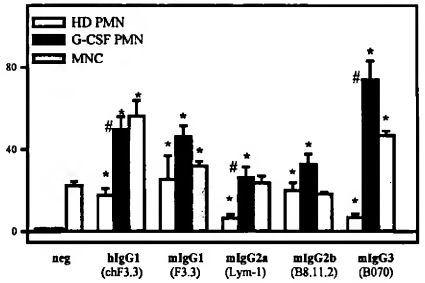

Fig. 2. Isotype dependency of MNC- and PMN cell-mediated ADCC. HLA class II antibodies of different isotypes were compared in ADCC against RAJI lymphoma cells using MNCs, healthy donor PMN cells (*HD PMN*), or G-CSF-primed PMN cells (*G-CSF PMN*) as effector cells. PMN cells mediated significant ADCC ($P < 0.05$, indicated by *) with all antibody isotypes, whereas MNCs were effective only with hIgG1, mIgG3, and mIgG1 isotypes. With antibodies of hIgG1, mIgG2a, and mIgG3 isotypes, G-CSF-primed PMN cells were significantly more cytotoxic ($P < 0.05$, indicated by #) than healthy donor PMN cells. Results from four experiments with different donors are displayed as mean ± SE of the percentage of specific lysis.

murine isotypes (Fig. 2). G-CSF-primed PMN cells were significantly more effective than healthy donor PMN cells with human IgG1, murine IgG2a, and murine IgG3 antibodies. From these data, we conclude that negative results with G-CSF-primed PMN cells and B-cell panel antibodies (Fig. 1) were not explained by insufficient interaction of sensitizing murine antibodies with human Fc receptors on PMN cells.

Isotype-dependent Involvement of FcγR in ADCC by G-CSF-primed PMN Cells. MNC-mediated ADCC was, as expected, completely blocked by $F(ab')_2$ fragments of FcγRIII antibody 3G8 (data not shown). In contrast to healthy donor PMN cells, which express both the low-affinity FcγRII (CD32) and FcγRIII (CD16), G-CSF-primed PMN cells additionally express the high-affinity FcγRI (CD64). The contribution of each of these three FcγR classes to ADCC was analyzed in assays via different target antibody isotypes by selectively blocking FcγRI, FcγRII, or FcγRIII with antibodies 197, IV.3, or 3G8, respectively (Fig. 3). Under our assay conditions, these Fc receptor antibodies are well documented to block selectively their respective Fc receptors (Refs. 13 and 16). Involvement of FcγRI was highest in assays via mIgG3, mIgA, and hIgG1 antibodies. These three isotypes were also more effective with G-CSF-primed PMN cells compared to healthy donor PMN cells (Fig. 2), indicating that expression of FcγRI on G-CSF-primed PMN cells causes enhanced killing via these isotypes. Blocking of FcγRII, on the other hand, had the most prominent influence in assays via mIgG1 and mIgG2b isotypes (in decreasing order). Interestingly, blockade of the most strongly expressed FcγRIII did not induce significant inhibition in assays via any of these target antibody isotypes, a finding that might relate to the glycosylphosphatidylinositol anchorage of FcγRIIIb in PMN cells. However, $F(ab')_2$ fragments of FcγRIII antibody 3G8 stimulated ADCC in FcγRII-dependent assays. In combination with data from Fig. 2, these results suggest that particularly the therapeutic

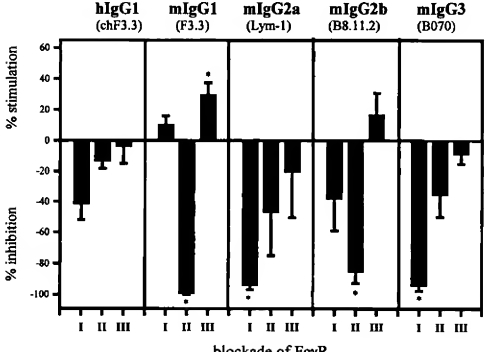

Fig. 3. Role of different FcγR in ADCC by G-CSF-primed PMN cells, depending on target antibody isotypes. G-CSF-primed PMN cells, expressing FcγRI, FcγRII, and FcγRIII, were used as effector cells in ADCC against RAJI lymphoma cells. Target cells were sensitized with HLA class II antibodies of different isotypes, and FcγR involvement was determined by blocking FcγRI (*I*), FcγRII (*II*), or FcγRIII (*III*) with MoAb 197 (whole antibody), IV.3 [F(ab') fragments], or 3G8 [F(ab')$_2$-fragments], respectively. Involvement of FcγRI, as indicated by blockade with MoAb 197, was highest with mIgG3 antibody (94 ± 4%), whereas blockade of FcγRII had the strongest influence on mIgG1-mediated ADCC (98 ± 1%). Blockade of FcγRIII showed no significant inhibition with any of the tested isotypes, but it stimulated FcγRII-mediated ADCC via mIgG1 and mIgG2b antibodies. Data represent mean ± SE of percentage of inhibition or stimulation from four experiments with different donors. *, significant influences of FcγR antibodies.

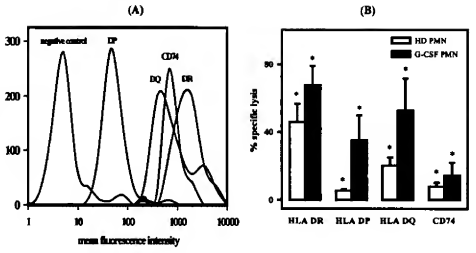

Fig. 4. Comparing HLA DR, DP, DQ and CD74 as target antigens on malignant B cells. *A*, antibodies against HLA DR (B8.11.2), DP (B7/21), DQ (SPV-L3), or CD74 (AT14/19) were used for sensitization of RM-1 mature B cells. *B*, in ADCC assays with PMN cells from healthy donors (*HD PMN*) or from G-CSF-treated patients (*G-CSF PMN*), both effector cell populations mediated significant killing ($P < 0.05$, indicated by *) by antibodies to all four target antigens. G-CSF-primed PMN cells were more effective than healthy donor PMN cells. Data from four experiments with different donors are presented as mean ± SE of the percentage of specific lysis.

efficacy of antibody isotypes reacting with FcγRI may benefit from a combination with G-CSF.

Comparing HLA DR, DP, DQ, and Invariant Chain (Ii, CD74) as Target Antigens. With the B-cell panel antibodies, PMN cells only mediated ADCC against malignant B-cells with HLA DR antibodies but not with antibodies to other B cell-related antigens (Fig. 1). Next, we analyzed whether PMN cells could induce B-cell lysis with antibodies to other HLA class II isoforms, or to the associated invariant chain (Ii, CD74). RM-1 mature B cells served as targets for these experiments because they were found to express high levels of all four antigens (Fig *4A*). As shown in Fig *4B*, both healthy donor and G-CSF-primed PMN cells induced significant ADCC with antibodies to HLA DR, DP, DQ, or via invariant chain antibodies. G-CSF-primed PMN cells were again more effective than healthy donor PMN cells.

To analyze whether neutrophil-mediated cytotoxicity by HLA class II antibodies is B cell specific, we used the HLA class II expressing T-cell line HUT-78 (RFI = 207) as the target for ADCC. In these experiments, healthy donor PMN cells were highly effective (40 ± 15% specific lysis; $n = 6$). Next, we tested whether HLA DR transfected into nonlymphoid cells can trigger ADCC in the presence of HLA class II antibodies. For this purpose, HLA DRα and DRβ were stably cotransfected into L cells (RFI = 55), which were then used as targets in ADCC. Again, effective ADCC was mediated by PMN cells in the presence of HLA class II antibody F3.3 (17 ± 7% specific lysis; $n = 4$).

Comparison of FcγRI-directed Bispecific Antibodies and Chimeric Human IgG1 Antibodies against HLA Class II and Its Variants Lym-1 and Lym-2. Results reported thus far were obtained with isolated effector cells at constant effector to target cell ratios. In addition to inducing FcγRI expression on PMN cells, *in vivo* application of G-CSF also dramatically increases PMN cell numbers. To assess the role of increased effector cell numbers, we established whole blood ADCC assays, in which 50 μl of freshly drawn blood from healthy donors or from patients receiving G-CSF were used as the effector sources. These assays, in addition to cell-mediated effects, measured antibody-mediated complement-dependent lysis. FcγRI-directed bispecific antibodies (all [F(ab') × F(ab')]) to classical HLA class II (22 × F3.3)), Lym-1 (22 × Lym-1), or Lym-2 (22 × Lym-2) antigens were compared with their respective mouse/human chimeric IgG1 constructs. ARH-77 mature B cells were used as targets in these assays because they expressed all three target antigens (Fig 5*A*). All three chimeric IgG1 constructs mediated significant lysis with blood from healthy donors (data not shown) or from G-CSF-treated patients (Fig 5*B*), especially at high antibody concentrations. However, there was no significant difference between healthy donor blood and blood from G-CSF-treated donors. Interestingly, whole blood from G-CSF-treated donors mediated ADCC with all three bispecific antibodies, and this was significantly higher than the cytotoxicity achieved with chimeric antibodies, especially with Lym-1- or Lym-2-directed bispecific antibodies (Fig 5*B*). As expected from the low numbers of FcγRI-expressing cells in healthy donors, FcγRI-directed bispecific antibodies were not effective with blood from healthy donors (data not shown).

When whole blood was then fractionated into plasma, MNCs, and PMN cells, the chimeric antibody against classical HLA class II was significantly more effective in inducing lysis with fresh human plasma than the Lym-1 or Lym-2 antibodies. This activity was completely abolished when plasma was heat inactivated, suggesting that complement activation was the underlying mechanism. Chimeric IgG1 antibodies directed against Lym-1 (data not shown) or Lym-2 (Fig. 6) mediated lysis mainly with mononuclear effector cells. However, FcγRI-directed bispecific antibodies also effectively recruited G-CSF-primed PMN cells and therefore were significantly more active with G-CSF-primed blood than chimeric IgG1 antibodies.

DISCUSSION

Results reported in this study extend previous observations that HLA class II antibodies are excellent in eliciting effector cell-mediated killing of malignant B cells (20, 27, 28). Antibodies to HLA class II were also reportedly effective in mediating complement-dependent lysis (29), in inhibiting cell proliferation (30), and in inducing apoptosis by Fas-mediated pathways (31), all of which are considered important effector mechanisms for therapeutic antibodies *in vivo*. HLA class II antigens are highly expressed on a broad spectrum of malignant B cells, are absent on hematopoietic stem cells and plasma cells, do not modulate, and are only found at low levels in soluble form. Furthermore, antibodies to HLA class II showed therapeutic efficacy against malignant B cells in syngeneic (32) and xenografted mouse tumor models (30, 33). Importantly, no side effects were observed in these mice, and extended immunological experiments did not reveal long-lasting immunosuppression after this treatment (32). However, HLA class II is not specific for malignant B cells but is also found on antigen-presenting cells like normal B cells, monocytes/macrophages, and dendritic cells. On these cells, HLA class II serves important functions in presenting antigens to CD4-positive T cells, and HLA class II antibodies were shown to induce tolerance under

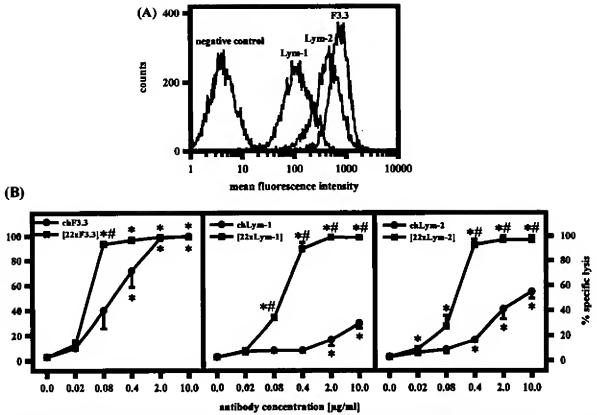

Fig. 5. Comparing chimeric IgG1 and FcγRI-directed bispecific antibodies against "classical" HLA class II and against HLA class II variants Lym-1, or Lym-2. *A*, whole blood from G-CSF-treated patients was used as effector source against ARH-77 mature B cells, which stained positive with HLA class II antibody F3.3, Lym-1, and Lym-2. *B*, significant lysis ($P < 0.05$, indicated by *) was observed with all six constructs. However, bispecific antibodies were significantly more effective ($P < 0.05$, indicated by #) than chimeric IgG1 antibodies, especially when Lym-1 or Lym-2 was targeted. Data from three experiments with different donors are presented as mean ± SE of the percentage of specific lysis.

certain experimental conditions (34). In addition, HLA class II expression is inducible on virtually every cell type by proinflammatory cytokines, such as IFN-γ. Subsequently, HLA class II antibodies showed severe toxicity in nonhuman primates, probably due to uncontrolled complement activation on cytokine-activated endothelial cells (35). These latter results have delayed the clinical development of HLA class II antibodies as therapeutic reagents because constructs with a lower complement-activating capacity were needed.

Reduced complement activation by MoAbs can be achieved by different approaches. For example, the C1Q-binding site of human IgG could be mutated, or antibody isotypes with less activity in complement activation, such as human IgG4 or human IgA, could be selected. Human IgA does not activate complement-dependent lysis, which seems beneficial when HLA class II is considered as target antigen, but is very effective in inducing cell-mediated lysis of tumor cells (19). Furthermore, antibodies to glycosylation variants of HLA class II, such as Lym-1 or Lym-2, have been shown to activate human complement (29) but were less potent than classical HLA class antibodies. Lym-1 and Lym-2 may have the additional advantage that they bind preferentially to HLA class II in malignant human B cells

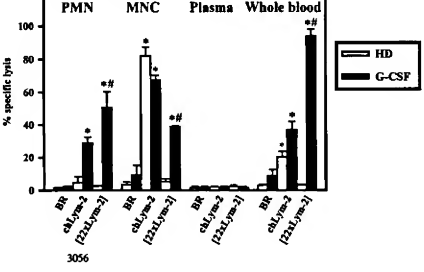

Fig. 6. Analyzing effector mechanisms of a chimeric IgG1 and an FcγRI-directed bispecific antibody against Lym-2. Lysis of ARH-77 mature B cells was measured comparing mouse/human chimeric IgG1 or (FcγRI × Lym-2) bispecific antibody directed to the Lym-2 antigen (both 2 μg/ml). As the effector source, whole blood from G-CSF-treated patients (*G-CSF*) or from healthy donors (*HD*) was compared and then fractionated into plasma, isolated MNCs or PMN cells. Interestingly, plasma was completely ineffective, indicating that no complement-mediated lysis occurred. In healthy donor blood, lysis with the chimeric IgG1 antibody resided mainly in the MNC fraction, whereas in G-CSF-treated patients, PMN cells were also recruited more effectively. As expected, the FcγRI-directed bispecific antibody was only effective with G-CSF-primed samples, in which it induced significantly higher killing ($P < 0.05$) than the chimeric antibody. *, significant lysis; #, differences between healthy donors and G-CSF-treated patients ($P < 0.05$).

compared to normal B cells and monocytes (23). A clinical Phase I trial with murine Lym-1 showed minimal toxicity in lymphoma patients (36). However, clinical responses with the unconjugated antibody were unsatisfactory, and the Lym-1 antibody is currently evaluated as a radioimmunoconjugate (37, 38). Meanwhile, antibodies Lym-1, Lym-2, and 1D10 (another antibody with similar binding characteristics) have been expressed as human IgG1 antibodies (30, 39), and clinical trials with these reagents are expected to start soon. However, in whole blood assays, effector cell recruitment by human IgG1 antibodies proved less optimal, as shown in Fig 5B.

Human IgG1 antibodies effectively activate human complement, interact well with FcγRIIIa on NK cells and macrophages (40), and have an extended half-life *in vivo* because they are protected from degradation by binding to FcRB (41). However, human IgG1 was less effective in recruiting PMN cells, the most populous Fc receptor-expressing effector cells in the blood. This could be explained by competition of therapeutic antibodies with high concentrations of natural immunoglobulins for binding to Fc receptors. This issue is especially critical for FcγRI because this high-affinity receptor binds monomeric IgG and therefore is not available as cytotoxic trigger molecule in the presence of serum concentrations of human IgG (42). In addition, therapeutic antibodies may bind to Fc receptors on non-effector cells (*e.g.*, platelets or B cells) or to Fc receptors on effector cells, which do not trigger cytolytic cascades (*e.g.*, FcγRIIb or FcγRIIIb). Fc receptor-directed bispecific antibodies represent an elegant solution to many of these problems (43). These genetically or chemically constructed molecules combine specificity for a tumor cell epitope with reactivity for a cytotoxic trigger molecule on immune effector cells, thereby allowing specific engagement of activating Fc receptors on cytotoxic cells. By selecting antibodies, which bind with their variable regions to Fc receptor epitopes distinct from the immunoglobulin binding site, competition with serum immunoglobulin can be avoided, and full activity in the presence of natural antibodies is conserved. Hematopoetic growth factors, such as G-CSF or granulocyte-macrophage CSF, can be used to dramatically enhance efficacy of Fc receptor-directed bispecific antibodies by increasing effector cell numbers and by up-regulating expression of select Fc receptors (44). Recently, we proposed FcαRI, which is found on monocytes/macrophages as well as eosinophilic and neutrophilic granulocytes, as promising trigger molecule (19). Bispecific antibodies directed against FcγRI (which is expressed on monocytes/macrophages, activated PMN cells, and subpopulations of dendritic cells) and FcγRIII (which is expressed on macrophages and NK cells) are currently being tested, with promising results, in clinical trials (43). In Phase I/II studies, Fc receptor-directed bispecific antibodies showed acceptable toxicity profiles and evidence of biological activity (45–48), leading to an ongoing evaluation for clinical efficacy (49). The results presented in this report provide the rationale for clinical studies with a combination of G-CSF- and FcγRI-directed bispecific antibodies to HLA class II-related antigens in lymphoma patients.

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Two New Monoclonal Antibodies, Lym-1 and Lym-2, Reactive with Human B-Lymphocytes and Derived Tumors, with Immunodiagnostic and Immunotherapeutic Potential[1]

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ABSTRACT

Two new monoclonal antibodies (Lym-1 and Lym-2), reactive with the cell surface of B-lymphocytes and derived tumors, have been produced using tumor cell nuclei preparations as immunogens. Specificity screens using live cell radioimmunoassay techniques with 52 well-characterized human lymphoma and leukemia cell lines showed that both Lym-1 and Lym-2 bound to cell lines of B-cell lineage but were unreactive with those of T-cell, myeloid, or erythroid derivation. The B-cell specificity of these reagents was confirmed on 36 lymphoma and 15 leukemia biopsy specimens by using immunoperoxidase or immunofluorescence techniques. Additionally, flow cytometric analysis of 22 lymphoma biopsies showed that the majority of B-cell tumors were Lym-1 and/or Lym-2 positive and that within a given biopsy, a high percentage of the malignant cell population was stained. In both the immunoperoxidase and flow cytometric studies, reactive T-cells or T-cell lymphomas were consistently negative with the exception of Hodgkin's disease tissues which, in some instances, showed a higher than expected positivity with Lym-1 and Lym-2. Approximately 40% of B-cell chronic lymphocytic leukemias were found to be positive with Lym-1 while 80% were positive with Lym-2. Immunoperoxidase staining of frozen sections of human lymphoid tissues showed that both Lym-1 and Lym-2 stained germinal center and mantle zone B-lymphocytes as well as interfollicular histiocytes. Flow cytometric analysis of normal peripheral blood demonstrated specific staining of B-cells which comprised approximately 8% of circulating lymphocytes. Immunoperoxidase staining of nonlymphoid human organs and tissues revealed weak reactivity of Lym-1 with surface colonic epithelium only. Consistent with these findings, 35 solid tumor cell lines of diverse nature were found unreactive with both Lym-1 and Lym-2. Although standard techniques have thus far failed to identify the antigen recognized by Lym-2, the membrane antigen which binds Lym-1 has been shown by immunoprecipitation and competitive radioimmunoassay studies to be a polymorphic variant of the HLA-DR antigen. Solid-phase radioimmunoassay techniques have shown that the antigens recognized by Lym-1 and Lym-2 are not significantly modulated after antibody exposure nor shed into the circulation of lymphoma patients. Finally, using Iodine-125 labeled preparations of purified Lym-1 and Lym-2, we have determined that both reagents have a relatively large number of antibody binding sites per tumor cell and increased avidity for lymphoma cells when compared to normal and reactive lymph node B-cells. Because of the B-cell specificity of these reagents, their increased avidity for lymphoma cells, and their chemical stability after radiolabeling procedures, Lym-1 and Lym-2 appear to be promising reagents for the immunodiagnosis and therapy of the human malignant lymphomas.

INTRODUCTION

Monoclonal antibodies to human B-cell antigens have been developed by a number of laboratories in the last several years. The majority of these reagents identify B-cell lineage-specific differentiation antigens such as CB2 (1); BL1, BL2, and BL3 (2, 3); BL7 (4); L22, L23, and L24 (5); HLB-1 (6); H-76 (7); B1 (8, 9); B2 (10); B4 (11); B5 (12); 4G7 (13); BA1 (14); HB-4 (15); anti-Y 29/55 (16); B532 (17); OKB1, OKB2, OKB4, and OKB7 (18); 41H.16 (19); and FMC7 (20). Others appear to be directed against lymphoma-specific antigens such as LM-26 and LM-155 (21), against Burkitt's lymphoma cells, or Epstein-Barr virus-transformed cell lines such as BB-1 (22) and BLA (23, 24) or against blast-associated antigens such as B-LAST-1 (25) and BLAST-2 (26). In addition, our laboratory has reported on three monoclonal antibodies, designated LN-1, LN-2, and LN-3 which are directed against B-cell antigens, but unlike similar reagents, retain their reactivity in B5-fixed, paraffin-embedded tissues and are therefore useful reagents for the immunodiagnosis of human lymphomas (27–29).

Most of these B-cell reagents have been shown by immunoperoxidase techniques to stain normal B-cells of reactive lymphoid tissues as well as lymphomas of B-cell derivation. Comparison studies by Hofman *et al.* (30), LeBien *et al.* (31), Marder *et al.* (28), Knowles *et al.* (32), and Gobbi *et al.* (33) have clearly shown that these monoclonal antibodies identify different antigens that are expressed on topographically distinct subsets of B-cells in human lymphoid tissues. Although originally thought to be B-cell specific, many of these reagents have now been shown to have reactivities in nonlymphoid tissues attributable to the presence of similar, if not identical, epitopes in related or different molecules (34). Furthermore, a thorough analysis of these monoclonal antibodies to determine their clinical utility has not been performed to date. Evaluation of these reagents with respect to their binding reactivity with a wide spectrum of human tumor cell lines and biopsy specimens, avidity constants and antibody binding site number, presence or absence of antigenic modulation and antigen shedding, antibody stability after purification and chemical conjugation procedures, and biodistribution in tumor-bearing hosts are required to identify those monoclonal antibodies destined for clinical trial. Despite the large number of anti-B-cell monoclonal antibodies described thus far, a single monoclonal antibody with immunotherapeutic potential has not been identified.

In this report, we describe the development and characterization of two new B-cell specific monoclonal antibodies, designated Lym-1 and Lym-2, which after careful analysis, appear to have the necessary properties to be successful *in vivo* immunodiagnostic and immunotherapeutic reagents. Other related studies describing the biodistribution of Lym-1 and Lym-2 in tumor-bearing nude mice and volunteer lymphoma patients, the enhancement of NMR[3] spin echo imaging of tumors in animal hosts, and the results of clinical trials involving the immunotherapy and radioimmunotherapy of end-stage lym-

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[3] The abbreviations used are: NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; cpm, counts per minute; BSA, bovine serum albumin.

phoma patients are being completed and will be presented elsewhere.

MATERIALS AND METHODS

Cell Lines and Tissues

A complete list of the cell lines used in these experiments is shown in Tables 1–3. Tumor cell lines designated with the SW prefix were obtained from Dr. William B. McCombs, III, at the Scott and White Memorial Hospital and Scott, Sherwood, and Brinkley Foundation, Temple, TX. All of the cell lines were grown in RPMI 1640 medium containing 15% fetal calf serum, penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml). The cell lines were cultured in a well-humidified 5% CO_2 incubator and were routinely passaged twice weekly.

For the immunohistochemical and flow cytometric studies, human tissues were obtained from biopsies performed on patients for diagnostic procedures at Northwestern University Memorial Hospital, Chicago, IL, and Los Angeles County–University of Southern California Medical Center, CA. Tissues for the frozen section studies were immediately snap frozen in liquid nitrogen and stored at −70°C until sectioning. Tissues for paraffin embedding were fixed in 10% buffered formalin or B5 formalin by using standard procedures. Tissues for flow cytometry were obtained either from normal volunteers for the peripheral blood experiments or from lymphoma patients undergoing diagnostic procedures.

Generation and Characterization of Hybridoma Clones

The methods for antigen preparation, immunization, cell fusion and cloning, and serological characterization of monoclonal antibody isotypes have been previously described in detail (27).

Immunofluorescence Techniques

Live cell indirect immunofluorescence techniques used in these experiments have been described previously (27).

Immunoperoxidase Staining Methods

Frozen and paraffin sections were prepared for immunoperoxidase staining procedures as described previously (27). For these experiments, a 1/2 dilution of Lym-1 and Lym-2 supernatants were used.

Flow Cytometric Studies

Lym-1 and Lym-2 were studied for their binding reactivity to human peripheral blood and malignant lymph node mononuclear cells by flow cytometric techniques. Lym-1 and Lym-2 binding to peripheral blood lymphocytes was assessed using indirect immunofluorescence staining with biotinylated anti-mouse IgG followed by phycoerythrin-conjugated avidin staining. Identification of B- and T-lymphocytes was performed using fluorescein isothiocyanate-conjugated B1 and OKT11 (Ortho) monoclonal antibodies, respectively, after completion of the phycoerythrin indirect labeling procedures with Lym-1 and Lym-2. This allowed the simultaneous assessment of Lym-1 and Lym-2 staining with anti-B (B1) or anti-T (OKT11) reagents with known reactivity (8, 35). The results were obtained using dual color flow cytometric techniques with a 525-nm band pass filter for the green photomultiplier tube and a 590-nm long pass filter for the red photomultiplier tube. Monocytes were assessed using phycoerythrin-conjugated Leu M3 (Becton Dickinson) (36) to establish the number of monocytes in the lymphocyte gate as well as the location of the monocyte population on forward angle and log90° light scatter modes. Lym-1 and Lym-2 binding reactivity was determined on both the monocyte and lymphocyte gates independently.

Malignant lymph nodes obtained at the time of surgery from 22 adult patients were prepared into single cell suspensions by passing the gently minced tissue through a stainless steel mesh screen. After Ficoll-Hypaque gradient centrifugation separation, the cells were stained by live cell indirect immunofluorescence techniques as previously described (37). The cells were analyzed cytofluorometrically by using an EPICS C (Coulter Corp., Hialeah, FL) flow cytometer.

Purification of Lym-1 and Lym-2 for Iodination Procedures

For the radioimmunoassay studies described below, it was necessary to obtain microgram quantities of both Lym-1 and Lym-2. Since highly purified reagent was necessary for the iodination procedures, it was decided to use tissue culture supernatant instead of ascites fluid as the source of Lym-1 and Lym-2. For Lym-1, Protein A-Sepharose affinity chromatography was the method of choice since Protein A has been shown to bind well to mouse IgG_{2a} (38). Consequently, 1 liter of filtered spent supernatant was mixed continuously overnight at 4°C with 1 g of preswollen Protein A-Sepharose CL-4B (Pharmacia, Inc., Piscataway, NJ). The beads were then washed extensively with phosphate buffered saline (8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.1 g $CaCl_2 \cdot 2H_2O$, and 0.1 g $MgCl_2 \cdot 6H_2O$/liter of distilled water) by centrifugation and aspiration to remove unbound material. After next placing the beads into a small column, the Lym-1 antibody was eluted into 1-ml fractions with 0.05 M sodium acetate and 0.15 M NaCl, pH 4.3. Fractions containing eluted antibody were determined by optical density at 280 nm. After pooling the fractions containing antibody, the preparation was dialyzed overnight against a ×1000 volume of PBS† at 4°C. The antibody was then sterile filtered and stored in aliquots (1 mg/ml) at 4°C.

The IgG_1 Lym-2 monoclonal antibody was purified from 1 liter of filtered spent supernatant batchwise as described above but with 1 g of Affi-gel Protein A and MAPS buffers as described by Biorad, Inc. After elution, the Lym-2 monoclonal antibody was dialyzed overnight at 4°C against a ×1000 volume of PBS, sterile filtered, and stored in aliquots (1 mg/ml) at 4°C.

The purity of the Lym-1 and Lym-2 preparations was checked by 10% polyacrylamide gel electrophoresis and by Ouchterlony immunodiffusion using immunoglobulin heavy chain specific antisera (Miles, Elkhart, IN). Protein concentrations of the purified preparations were determined by optical density at 280 nm and by the Biorad Protein Assay Kit (Biorad).

Radioimmunoassay Methods

Live Cell Radioimmunoassay. Human malignant lymphoma, leukemia, and solid tumor cell lines were assessed for Lym-1 and Lym-2 binding using a live cell radioimmunoassay method. Briefly, suspension cultures and solid tumor cell lines which were dislodged from their flasks with EDTA-trypsin (Grand Island Biological Co., Grand Island, NY), were washed twice in cold buffer consisting of PBS, bovine serum albumin (1 mg/ml, radioimmunoassay grade; Sigma Chemical Co., St. Louis, MO), and 0.02% sodium azide. Cells (5×10^5) resuspended in 100 μl of wash buffer were pipeted into microtiter wells (Immulon Removawell Strips; Dynatech Laboratories, Inc., Alexandria, VA). The microtiter plates were pretreated the previous night with bovine serum albumin (10 mg/ml) in PBS with azide in order to prevent the antibody solutions from binding to the wells. Hybridoma supernatant (100 μl) was added to each well, and the plates were incubated for 30 min at room temperature with continuous mixing using a microshaker apparatus (Dynatech) set at low speed. After incubation, the cells were washed 4 times with cold wash buffer by spinning the plates at 1,000 rpm for 5 min, aspirating the supernatants with a 12-tip micromatic manifold (Popper and Sons, Inc., New Hyde Park, NY), and resuspending the cells in 200 μl of wash buffer using a Titertek Multichannel pipet (Flow Laboratories, Inc., McLean, VA) and the microshaker apparatus. After completion of the washes, 100,000 cpm of [^{125}I]goat anti-mouse serum (Amersham Corp., Arlington Heights, IL) in a volume of 100 μl, were added to each well for an additional 30-min incubation period with continuous shaking. Finally, the cells were washed 4 times as above and the wells were counted in a gamma counter at 1-min intervals. All data were expressed as the mean cpm of triplicate samples minus that of the NS-1 supernatant control wells. For each test, the standard deviation of the triplicate cpm was calculated and, if found to be greater than 10% of the mean cpm, the assay was repeated.

Detection of Shed Antigen. The presence of shed antigen was detected

using a sensitive sandwich solid-phase radioimmunoassay developed in our laboratory. Purified Lym-1 or Lym-2 at a concentration of 1 mg/ml in PBS was added to flat-well Immulon plates using a 50-μl volume per well. After a 1-h incubation at room temperature, the wells were washed once to remove unbound antibody. All washes were performed with 200 μl of BSA (1 mg/ml) in PBS containing 0.02% sodium azide. The wells were then blocked for 30 min with BSA (10 mg/ml) in PBS, washed twice, and incubated with 50 μl of either target cell membrane preparations, target cell supernatant concentrated ×25 in B15 Minicon Concentrators (Amicon Corp., Danvers, MA), or serum samples from lymphoma patients. The target cell lines used were Raji for Lym-1, ARH-77 for Lym-2, and CEM T-acute lymphoblastic leukemia as the negative control. After a 1-h incubation at room temperature, the wells were washed 3 times and 100,000 cpm/well of [^{125}I]sheep anti-mouse reagent were added to each well in a volume of 50 μl/well. After a final 1-h incubation period at room temperature, the wells were washed 5 times and counted in a gamma counter.

Antigenic Modulation. One million Raji cells (for Lym-1) or ARH-77 cells (for Lym-2) were incubated at time 0 with 100 μl of increasing concentrations of purified unlabeled Lym-1 or Lym-2 for 30 min at room temperature with continuous mixing. After removing unbound antibody by centrifugation, the cells were incubated for 24 and 48 h in complete RPMI 1640 medium at 37°C in a 5% CO_2 humidified incubator at which time 100 μl of 10^5 cpm of [^{125}I]Lym-1 or [^{125}I]Lym-2 were incubated with the cells for 30 min at room temperature with continuous mixing. After three washes, to remove unbound reagent, the cells were counted in a gamma counter. Iodine-125-labeled Lym-1 and Lym-2 monoclonal antibodies were prepared by a modified chloramine-T method as described below.

Quantitation of Antibody Avidity and Binding Site Number

In order to determine the avidity constant and binding site number of Lym-1 and Lym-2, a live cell radioimmunoassay was performed using directly labeled antibody preparations. The binding constants and number of antibody binding sites were determined by the methods and equations described by Frankel and Gerhard (39). Purified Lym-1 and Lym-2 were radioiodinated using a modified chloramine-T method (40). Briefly, 50–100 μg of antibody in 50–100 μl was added to 400 μCi of iodine-125 (4 μl) (New England Nuclear Research Products, Boston, MA) in a test tube. Chloramine-T was then added to the mixture and the reaction vessel was covered and incubated at room temperature for 90 s with constant shaking. The ratios of chloramine-T:Lym-1 and chloramine-T:Lym-2 were 1:4.0 and 1:4.6 (w:w), respectively. At the end of the incubation, the reaction was terminated by adding sodium metabisulfate at a 2:1 molar ratio to chloramine-T. The iodinated antibody was then separated from free iodine by standard methods using gel filtration. Fractions of labeled product were stored at 4°C until use. The protein concentration of the radiolabeled antibodies were determined by the BioRad Protein Assay using bovine serum albumin as the standard. Immunoreactivity defined as the percentage of labeled antibody still capable of binding to the target cell lines was assessed by live cell radioimmunoassay described above.

For the binding constant experiments, the concentration of antibody was varied by dilution with PBS. Each experimental variable was run in triplicate. Washed target cell suspensions containing 5×10^5 cells were added to each well of a 96-well Immulon microtiter plate which was previously blocked overnight with BSA (10 mg/ml) in PBS. The cells were incubated with 200 μl of PBS containing 0.5–500 ng of radiolabeled Lym-1 or Lym-2 for 1 h at room temperature with constant shaking. Cells were then washed ×3 with PBS containing bovine serum albumin (1 mg/ml) to remove unbound antibody and counted in a gamma counter. The amount of bound antibody was then determined by the radioactivity (cpm) in each well and the specific activity (cpm/ng) of the radiolabeled monoclonal antibody. For comparative purposes, parallel experiments using freshly prepared tonsil lymphocytes were also performed. The tonsil mononuclear cell preparation was prepared in the same manner as the malignant lymph nodes described above.

Live Cell Competition Studies. Competitive live cell radioimmunoassay procedures were performed with Lym-1 and anti-Dr and anti-Ds monoclonal antibodies (Mallinckrodt, Inc., St. Louis, MO) in order to determine the relationship of Lym-1 to HLA class II-related antigens expressed on human lymphoma cells. For these studies, 10^6 Raji cells per tube were washed twice with BSA (1 mg/ml) in PBS containing 0.02% sodium azide. The cells were then incubated at 4°C with 200 μl/tube of serial dilutions of anti-Dr and -Ds monoclonal antibodies for 30 min with constant mixing. After incubation, the cells were washed twice and 100,000 cpm/tube of [^{125}I]Lym-1 in a volume of 100 μl/tube were added for an additional 30-min incubation period. The cells were then washed twice and counted in a gamma counter.

Immunoprecipitation and Immunoblot Studies

Raji cells and ARH-77 myeloma cells were used in immunoprecipitation and immunoblot studies as previously described (27, 41) in order to identify the molecular weight of the antigens recognized by Lym-1 and Lym-2.

RESULTS

Generation of Monoclonal Antibodies Lym-1 and Lym-2. Hybridoma clone Lym-1 was produced by the fusion of mouse myeloma NS-1 cells and BALB/c splenocytes obtained from a mouse hyperimmunized with nuclei from Raji Burkitt's lymphoma cells. Isotypic analysis revealed that monoclonal antibody Lym-1 is of the IgG_{2a} heavy chain subclass. Hybridoma clone Lym-2 was produced as above from a mouse hyperimmunized with chronic lymphocytic leukemia cell nuclei. Isotypic analysis of supernatant revealed that Lym-2 is of the IgG_1 heavy chain subclass. Both monoclonal antibodies were initially identified by indirect immunofluorescence techniques with the use of paraformaldehyde-acetone-fixed cell preparations where they gave a speckled membrane (Lym-1) or membrane ring (Lym-2) pattern of staining. Both Lym-1 and Lym-2 have been subcloned on agar plates and have retained their ability to secrete antibody (2–10 μg/ml) in a stable manner for more than 2 yr. Both hybridomas have been adapted to grow exponentially in serum-free medium (Nutraclone-M; Techniclone International, Inc., Santa Ana, CA) with the same (Lym-1) or slightly slower (Lym-2) growth rates as cultures grown in 10% fetal calf serum facilitating their use in fermentation or hollow fiber culture equipment.

Binding Reactivity of Lym-1 and Lym-2 with Established Human Neoplastic Cell Lines by Live Cell Radioimmunoassay. The binding reactivities of monoclonal antibodies with established human malignant lymphoma, leukemia, and solid tumor cell lines are shown in Tables 1, 2, and 3, respectively. For these studies, a live cell radioimmunoassay method was used in order to directly assess the cell surface binding reactivity of these monoclonal antibodies with a wide spectrum of tumor cell lines. Both monoclonal antibodies appear unrestricted in their reactivity to human lymphomas and leukemias of B-cell origin.

Immunoperoxidase Staining Reactivity of Lym-1 and Lym-2 with Human Lymphoid and Non-Lymphoid Tissues. In order to assess the binding reactivity of Lym-1 and Lym-2 with normal human tissues, frozen sections of lymphoid and nonlymphoid organs from surgical biopsy material were stained with Lym-1 and Lym-2 supernatants by the avidin-biotin immunoperoxidase technique. As shown in Table 4 and in Fig. 1, both monoclonal antibodies reacted with the cell membrane of B-lymphocytes in human lymph node sections. Lym-1 showed a speckled membrane pattern and reacted principally with germinal center and mantle zone B-cells and interdigitating histiocytes scattered in the T-cell zones. Lym-2 gave a membrane ring pattern of staining and was positive on both germinal

Table 1 *Reactivity of Lym-1 and Lym-2 with human malignant lymphoma cell lines by live cell radioimmunoassay*

Cell line	Lym-1	Lym-2
Burkitt's lymphoma		
Raji	++++[a]	++
EB3	–	–
DG-75	++++	++++
NK-9	++	++++
AL-1	–	+
Daudi	+	+++
NU-AmB-1	+	++
SU-AmB-1	–	+
SU-AmB-2	–	–
RAMOS	–	–
Chevallier	++++	–
B46M	+	+
B35M	++++	++++
DND-39	+	–
U-698-M	+	++
HRIK	–	+
Large cell lymphoma		
SU-DHL-1	–	–
SU-DHL-2	–	–
SU-DHL-4	–	++++
SU-DHL-5	+	++
SU-DHL-6	+++	+++
SU-DHL-7	+	–
SU-DHL-8	+	–
SU-DHL-9	+	+
SU-DHL-10	–	++++
SU-DHL-16	–	–
NU-DHL-1	++++	–
U-937	–	–
Undifferentiated lymphoma		
NU-DUL-1	–	+

[a] –, <2,000 cpm; +, 2,000–6,000 cpm; ++, 6,000–10,000 cpm; +++, 10,000–15,000 cpm; ++++, >15,000 cpm.

Table 2 *Reactivity of Lym-1 and Lym-2 with human leukemia and lymphoblastoid cell lines by live cell radioimmunoassay*

Cell line	Lym-1	Lym-2
Acute lymphoblastic leukemia		
T-cell		
MOLT-4	–[a]	–
CEM	–	–
HSB-2	–	–
HPB-ALL	–	–
JM	–	–
Null cell		
REH	+	++
NALL-1	–	–
KM-3	–	–
L92-221	–	–
Pre-B-cell		
NALM-1 (from CML)	–	–
NALM-6	+	–
BALM-2	+++	++
BALM-5	–	–
B cell		
BALL-1	+++	+
Myeloid leukemia		
HL-60 (promyelocytic)	–	–
ML-2 (myeloid)		–
KG-1 (myeloid)	–	–
TPH-1-0 (monocytic)	–	–
K562 (erythroid CML)	–	–
HEL 92.1.7 (erythroid)	–	–
Myeloma		
U-266	–	–
ARH-77	+++	++++
HS Sultan	+++	+++
Lymphoblastoid		
BL-1	–	+
NU-LB-1	+++	++++
NU-LB-2	++	++
ERIC-LB-2	+++	+
ERIC-LB-3	+	++

[a] –, <2,000 cpm; +, 2,000–6,000 cpm; ++, 6,000–10,000 cpm; +++, 10,000–15,000 cpm; ++++, >15,000 cpm.

center and mantle zone B-lymphocytes. T-lymphocytes, sinus histiocytes, and endothelial cells of reactive lymph nodes and tonsils were not stained by both Lym-1 and Lym-2. In the thymus, Lym-1 was found to react with medullary dendritic cells, and in the spleen, both reagents stained B-cells of the white pulp. In multiple samples of normal bone marrow, both Lym-1 and Lym-2 were negative with myeloid, erythroid, and megakaryocyte precursor and mature cells.

As shown in Table 5, both Lym-1 and Lym-2 did not demonstrate significant binding reactivity with a large panel of normal human tissues obtained at biopsy. On some but not all specimens of human colon, Lym-1 did show weak staining reactivity with the epithelium of the luminal surface. Lym-1 was also positive with macrophages in the skin. Lym-2 was found completely unreactive with all tissues tested to date.

Staining Reactivity of Lym-1 and Lym-2 with Human Malignant Lymphoma and Leukemia Biopsy Specimens. As shown in Table 6, Lym-1 and Lym-2 were reacted with frozen sections from 36 lymphomas obtained at biopsy. Immunoperoxidase staining with Lym-1 and Lym-2 demonstrated significant and strong positivity with the majority of B-lymphomas. The T-cell lymphomas were unreactive with both antibodies even though they both were positive for the HLA-Dr antigen (data not shown). Lym-1 showed greater positivity with the large cell lymphomas and less reactivity with the small lymphocytic lymphomas while Lym-2 was found to react equally well with all types of B-lymphomas. An example of the immunoperoxidase staining reactivities of Lym-1 and Lym-2 on lymphoma tissue is presented in Fig. 2. As shown, this intermediate-grade lymphoma biopsy specimen had stronger reactivity with Lym-2 than Lym-1.

Lym-1 and Lym-2 were also reacted with 15 cases of chronic lymphocytic leukemia. As shown in Table 6, Lym-1 had a 40% reactivity with B-cell cases and no reactivity with all five T-cell chronic lymphocytic leukemias. Lym-2 showed an 80% reactivity with B-cell cases and likewise was unreactive with T-cell leukemias. These results indicate that both Lym-1 and Lym-2 are reactive with the majority of B-cell lymphomas and leukemias and are restricted in their binding to B-cell derived tumors.

Flow Cytometric Analysis of Lym-1 and Lym-2 Reactivity with Peripheral Blood Cells and Malignant Lymph Node Biopsy Specimens. In order to assess in a quantitative manner the type and percentage of Lym-1 and Lym-2 positive cells in normal peripheral blood mononuclear cells, flow cytometric studies were performed. In all cases studied, Lym-1 and Lym-2 were found unreactive with T-cells, Leu M3 positive monocytes, granulocytes, red blood cells, and platelets. B-lymphocytes, however, were found reactive with both Lym-1 and Lym-2 as demonstrated in Fig. 3 where dual labeling studies with the pan-B monoclonal antibody B1 showed simultaneous B1 and Lym-1 (Fig. 3*C*) and B1 and Lym-2 (Fig. 3*F*) reactivity. Quantitation of these results shows that for Lym-1, 90% of $B1^+$ cells are $Lym\text{-}1^+$ while 10% of $B1^+$ cells are $Lym\text{-}1^-$. For Lym-2, 86% of $B1^+$ cells are $Lym\text{-}2^+$ while 14% of $B1^+$ cells are $Lym\text{-}2^-$. Conversely, 10% of $B1^-$ cells are $Lym\text{-}1^+$ and 1% of $B1^-$ cells are $Lym\text{-}2^+$. These results indicate that roughly 8% of circulating B-lymphocytes found in normal peripheral blood samples are both Lym-1 and Lym-2 positive. The reactivities of monoclonal antibodies B1, Lym-1, and Lym-2, however, do show some differences indicating that small subpopulations of B-cells have varying reactivities with these B-cell reagents. As

Table 3 *Reactivity of Lym-1 and Lym-2 with 35 human solid tumor cell lines by live cell radioimmunoassay*

Cell line	Derivation	Lym-1	Lym-2
734B	Breast carcinoma	–[a]	–
578T	Breast carcinoma	–	–
C-399	Colon carcinoma	–	–
Hutu-80	Colon carcinoma	–	–
HT-29	Colon carcinoma	–	–
HeLa	Cervical carcinoma	–	–
SW 733	Papillary carcinoma of bladder	–	–
SW 780	Transitional cell carcinoma of bladder	–	–
SW 451	Squamous cell carcinoma of esophagus	–	–
SW 579	Squamous cell carcinoma of thyroid	–	–
SW 156	Hypernephroma	–	–
60	Small cell carcinoma of lung	–	–
464	Small cell carcinoma of lung	–	–
NCI-H69	Small cell carcinoma of lung	–	–
125	Adenocarcinoma of lung	–	–
A427	Adenocarcinoma of lung	–	–
A549	Adenocarcinoma of lung	–	–
SW 1503	Mesothelioma	–	–
BM 166	Neuroblastoma	–	–
IMR-5	Neuroblastoma	–	–
Y79	Retinoblastoma	–	–
A172	Astrocytoma	–	–
SW 608	Astrocytoma	–	–
U118 MG	Glioblastoma	–	–
NU-04	Glioblastoma	–	–
CaCl 74-36	Melanoma	–	–
Colo 38	Melanoma	–	–
SW 872	Liposarcoma	–	–
HS 919	Liposarcoma	–	–
SW 1045	Synovial sarcoma	–	–
SW 80	Rhabdomyosarcoma	–	–
SW 1353	Chondrosarcoma	–	–
4-998	Osteogenic sarcoma	–	–
4-906	Osteogenic sarcoma	–	–
SU-CCS-1	Clear cell sarcoma	–	–

[a] –, <2,000 cpm; +, 2,000–6,000 cpm; ++, 6,000–10,000 cpm; +++, 10,000–15,000 cpm; ++++, >15,000 cpm.

Table 4 *Reactivity of Lym-1 and Lym-2 with lymphoid and hematopoietic tissues in frozen sections or cytospins*

Organ	Lym-1	Lym-2
Lymph node		
Germinal center	+++[a]	++
Mantle zone	+	+++
T-cell zones	–	–
Interdigitating histiocytes	++	++
Sinus histiocytes	–	–
Endothelium	–	–
Thymus		
Cortex	–	–
Medulla	++ Dendritic cells	–
Spleen		
White pulp	++ B-cell zones	++ B-cell zones
Red pulp	–	–
Bone marrow		
Myeloid	–	–
Erythroid	–	–
Megakaryocytes	–	–

[a] Intensity of immunoperoxidase staining ranging from – to +++.

shown in Table 7, flow cytometric analysis of 22 consecutive malignant lymphoma biopsy specimens revealed that Lym-1 and Lym-2 stained the majority of B-cell cases. OKT11 positive T-cell lymphomas (*cases 7* and *16*) were negative with both reagents. Lym-1 and Lym-2 showed similar but not identical staining reactivity and for individual cases, Lym-2 usually had a higher percentage of positive cells. For some of the Hodgkin's disease cases, such as *case 22*, there was an unusual overlapping of positivity with both Lym-1 and Lym-2 and OKT11, indicating that some T-cell marker positive cells were also expressing B-cell antigens. Further Hodgkin's disease cases need to be studied in order to determine the significance of these findings. In general, these data confirm the immunoperoxidase results reported above.

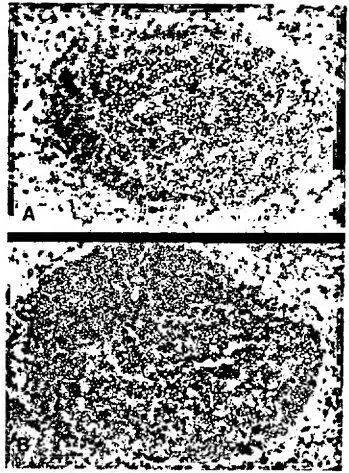

Fig. 1. Immunoperoxidase staining of Lym-1 and Lym-2 monoclonal antibodies with germinal center and mantle zone B lymphocytes in frozen sections of human tonsil. *A*, Lym-1; *B*, Lym-2 (× 325).

Table 5 *Reactivity of Lym-1 and Lym-2 with normal nonlymphoid tissues in frozen sections*

Tissue	Reactivity	
	Lym-1	Lym-2
Adrenal	–[a]	–
Brain	–	–
Breast	–	–
Cervix	–	–
Colon	+ surface epithelium	–
Duodenum	–	–
Heart	–	–
Kidney	–	–
Liver	–	–
Lung	–	–
Ovary	–	–
Pancreas	–	–
Salivary glands	–	–
Skin	+ macrophages only	–
Skeletal muscle	–	–
Smooth muscle	–	–
Stomach	–	–
Testis	–	–
Thyroid	–	–

[a] Intensity of immunoperoxidase staining ranging from – to +++.

Radioimmunoassay Results of Antigen Shedding and Antigenic Modulation. The results of the antigen shedding and antigenic modulation studies are presented in Tables 8 and 9, respectively. These studies demonstrated that the antigen recognized by Lym-1 on human lymphoma cells is neither shed nor modulated after antibody binding. Sensitive solid-phase radioimmunoassay

Table 6 *Reactivity of Lym-1 and Lym-2 with human malignant lymphoma and leukemia biopsy specimens*

Diagnosis	Lym-1[c]	Lym-2[c]
Lymphomas[a] (frozen sections of lymph node biopsies[b])		
Well-differentiated lymphocytic	1/3	3/3
Poorly differentiated lymphocytic, nodular	0/2	2/2
Poorly differentiated lymphocytic, diffuse	1/3	3/3
Mixed lymphocytic and histiocytic	8/9	7/9
Histiocytic (B-cell)	12/17	12/17
T-cell	0/2	0/2
Leukemias (cytospins of peripheral blood[d])		
Chronic lymphocytic		
B-cell type	4/10	8/10
T-cell type	0/5	0/5

[c] Positive/total.
[b] Rappaport classification.
[c] Immunoperoxidase technique.
[d] Indirect immunofluorescence.

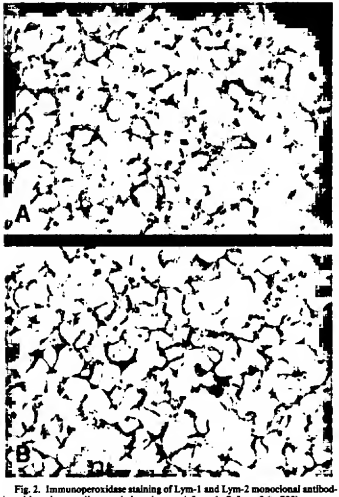

Fig. 2. Immunoperoxidase staining of Lym-1 and Lym-2 monoclonal antibodies with an intermediate grade lymphoma. *A*, Lym-1; *B*, Lym-2 (× 720).

methods also failed to detect circulating antigen in the serum of antigen positive lymphoma patients at the time of diagnosis. Although no antigen shedding was observed with Lym-2, partial modulation of the Lym-2 antigen was observed at high antibody concentrations (0.01 mg and higher).

Avidity Constant and Binding Site Number of Lym-1 and Lym-2 on Target Lymphoma Cell Lines and Reactive Tonsil B Lymphocytes. In Fig. 4, the binding profiles of radiolabeled Lym-1 and Lym-2 with Raji and ARH-77 tumor cells, respectively, are shown. With a large excess of antibody present, the equilibrium of the antigen-antibody reaction is expected to be driven in the direction of the antigen-antibody complex. Therefore, it is assumed that each determinant of antigen on the cell surface has bound with antibody at saturation. By this assumption, the number of binding sites per cell can be calculated by Equation A:

$$\frac{(\text{ng of Ab}) \times 10^9 \times N}{\text{Molecular weight of Ab} \times \text{number of cells}} \qquad \text{(A)}$$

where N is Avogadro's Number and "ng of Ab" is the amount of antibody bound to the cell at saturation. The latter value was calculated from the cpm per well obtained by radioimmunoassay and the specific activity (cpm/ng) of the radiolabeled monoclonal antibody. Based upon these experiments, the number of binding sites per Raji Burkitt's lymphoma cell with Lym-1 was found to be 1.1×10^6, while the number of binding sites per ARH-77 cell with Lym-2 was 2.0×10^5.

As shown in Figs. 5 and 6, a Scatchard plot analysis of the radioimmunoassay data for Lym-1 and Lym-2 using Raji and ARH-77 cells, respectively, was used to obtain linear regression curves in order to calculate the slope. From the slope, the equilibrium or avidity constant K was calculated by the equation, $K \approx -(\text{slope}/n)$.

In Table 10 the avidity constants for Lym-1 and Lym-2 on target tumor cells lines and reactive tonsil lymphocytes are shown. A greater than 4-fold difference in K value for Lym-1 and approximately a 2-fold difference in K value for Lym-2 was obtained when tumor cell and tonsil lymphocytes were compared. These data indicate that both monoclonal antibodies have an increased avidity of binding for lymphoma cells as compared to normal B-lymphocytes.

Immunobiochemical Characterization of Lym-1 and Lym-2 Antigens. Immunoblot methods using denatured protein preparations failed to identify the molecular weight of the antigens recognized by Lym-1 and Lym-2. Immunoprecipitation techniques with metabolically labeled protein lysates did, however, identify the antigen recognized by Lym-1 as shown in Fig. 7. Using Raji cells as the source of radiolabeled proteins, 4 polypeptide bands with molecular weights of 31,000, 32,000, 34,000, and 35,000 were immunoprecipitated in a consistent manner by Lym-1 supernatant. Since this protein was so similar in molecular weight to the HLA-Dr antigen, side-by-side immunoprecipitation studies with Lym-1 and the anti-HLA-Dr monoclonal antibody SC2 (gift of Dr. Robert Fox, Scripps Clinic and Research Foundation) were performed. In Fig. 7, these immunoprecipitation results are shown with Raji cell lysates. The immunoprecipitation pattern obtained with Lym-1 was quite similar to that obtained with SC2 but differed in two respects. First, all of the bands identified in the SC2 immunoprecipitate are not seen in the Lym-1 immunoprecipitate indicating that Lym-1 only recognizes part of the HLA-Dr antigen or recognizes a polymorphic variant of the molecule. Second, a 3-week exposure for Lym-1 compared to a 10-day exposure for SC2 was required for the autoradiographs to be adequately visualized. These results may indicate that Lym-1 is seeing only a less abundant variant of the HLA-Dr molecule. To further test the relationship of Lym-1 to the HLA-Dr antigen, competition studies with Lym-1 and monoclonal anti-HLA-Dr and -Ds reagents were performed. As shown in Table 11, the anti-HLA-Ds monoclonal antibody was not able to block Lym-1 binding but the anti-HLA-Dr reagent successfully blocked Lym-1 binding to Raji cells even at low concentrations. These results confirm the immunoprecipitation results and suggest that Lym-1 is recognizing the HLA-Dr antigen or a closely related variant of this molecule.

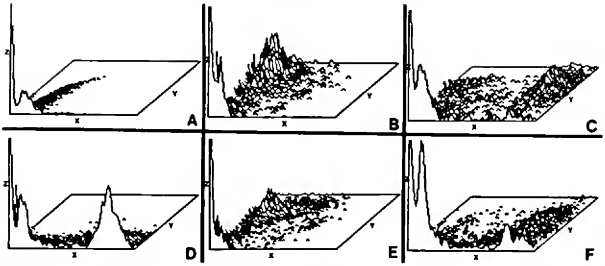

Fig. 3. Flow cytometric analysis of Lym-1 and Lym-2 staining reactivity with normal peripheral blood lymphocytes. *A*, negative control; *B*, Lym-1; *C*, Lym-1 and B1; *D*, B1; *E*, Lym-2, and *F*, Lym-2 and B1.

Table 7 *Flow cytometric analysis of malignant lymphoma biopsy specimens using Lym-1 and Lym-2 and lymphocyte markers*

Case	Sex	Age (yr)	Diagnosis (working formulation)	Biopsy site	OKT11	Sig	κ	λ	Lym-1	Lym-2
High grade lymphomas										
1	F	41	Small noncleaved, diffuse	L inguinal LN[a]	3	5	1	0	26	43
2	M	60	Small noncleaved, diffuse	R cervical LN	11	46	2	65	36	37
3	F	86	Small noncleaved, diffuse	Chest wall	26	67	36	0	2	0
4	M	47	Small noncleaved, diffuse	R inguinal LN	6	96	1	96	20	28
5	F	54	Immunoblastic	L submental LN	62	12	8	3	38	30
6	M	50	Immunoblastic	R axillary LN	55	82	18	18	80	70
7	F	89	Immunoblastic	L subclavian LN	96	25	21	1	12	17
Intermediate grade lymphomas										
8	F	73	Follicular, large cell	L axillary LN	18	87	87	7	57	84
9	F	31	Small cleaved, diffuse	Conjunctiva	8	95	7	85	51	75
10	M	87	Large cell, diffuse	R inguinal LN	14	23	9	1	86	61
Low grade lymphomas										
11	F	69	Follicular, small cleaved	L inguinal LN	16	38	2	34	76	80
12	M	53	Follicular, small cleaved	Small bowel	22	83	81	2	78	83
13	F	56	Follicular, small cleaved	L inguinal LN	26	100	79	2	85	90
14	F	39	Follicular, small cleaved	L inguinal LN	4	88	2	83	30	59
15	F	76	Follicular, mixed	R ovary	27	67	60	9	23	47
16	M	70	Follicular, large cell	L inguinal LN	61	10	7	5	8	31
17	M	55	Small lymphocytic (CLL)	Spleen	2	95	95	1	32	77
Hodgkin's disease										
18	M	38	HD/L-H	Cervical LN	39	72	63	64	69	86
19	F	15	NSHD	R cervical LN	81	20	17	8	7	24
20	F	18	NSHD	L inguinal LN	94	6	4	2	7	9
21	M	66	NSHD	L cervical LN	59	31	20	10	21	37
22	M	35	HD/mixed cellularity	R cervical LN	97	3	1	1	48	49

[a] LN, lymph node; HD, Hodgkin's disease; NSHD, nodular sclerosis Hodgkin's disease; HD/LH: Hodgkin's disease, lymphocytic-histiocytic type; L, left; R, right. CLL, chronic lymphocytic leukemia.

Immunoprecipitation studies with Lym-2 using ARH-77 radiolabeled lysates have to date yielded negative results. Additional experiments to identify the antigen recognized by Lym-2 are being conducted.

DISCUSSION

Two new monoclonal antibodies, Lym-1 and Lym-2, specific to normal and malignant human B-cells have been produced. The salient features of these antibodies are summarized in Table 12. Both monoclonal antibodies were developed from mice hyperimmunized with tumor cell nuclei as part of other experiments. Although these experiments were directed at producing reagents specific for nuclear constituents, we identified a handful of monoclonal antibodies with high avidity for the cell surface, which we assume recognized small remnants of cell

Table 8 *Detection of shed antigen by radioimmunoassay*

Antigen source	Radioimmunoassay	[^{125}I]Lym-1 (cpm)	[^{125}I]Lym-2 (cpm)
Target cells (1×10^6)	Live cell	51,582	40,105
CEM cells (1×10^6)	Live cell	214	456
Target membranes	Solid phase, direct	6,060	2,418
Target membranes	Solid phase, indirect	19,274	5,350
Target cell supernatant[a]	Solid phase, indirect	619	621
Lymphoma patient sera (no.)			
1	Solid phase, indirect	115	291
2	Solid phase, indirect	111	263
3	Solid phase, indirect	168	195
4	Solid phase, indirect	385	280
5	Solid phase, indirect	269	256

[a] Concentrated × 25.

Table 9 *Antigenic modulation studies with Lym-1 and Lym-2*

Concentration of Lym-1 and Lym-2 used to bind 10^6 target cells at 0 h	[^{125}I]Lym-1 (cpm)		[^{125}I]Lym-2 (cpm)	
	24 h	48 h	24 h	48 h
0.1 mg	18,444	32,938	3,662	4,850
0.05 mg	26,715	33,475	7,515	10,237
0.01 mg	31,502	40,169	11,008	17,105
0.005 mg	31,636	43,463	14,240	25,967
0.001 mg	37,665	46,928	19,776	27,116
PBS control	43,946	47,600	33,152	32,216

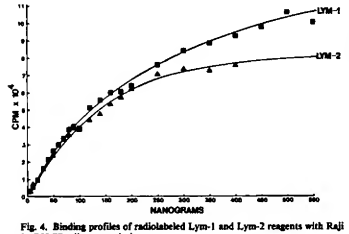

Fig. 4. Binding profiles of radiolabeled Lym-1 and Lym-2 reagents with Raji and ARH-77 cells, respectively.

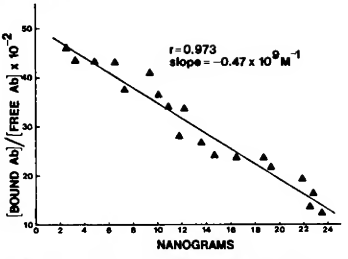

Fig. 6. Scatchard plot analysis of Lym-2 binding data with ARH-77 cells.

Table 10 *Avidity constants of Lym-1 and Lym-2 using target tumor cell lines and tonsil lymphocytes*

Monoclonal antibody	Tumor cell line	Tonsil
Lym-1	4.02×10^8 M^{-1}	0.88×10^8 M^{-1}
Lym-2	2.33×10^8 M^{-1}	1.23×10^8 M^{-1}

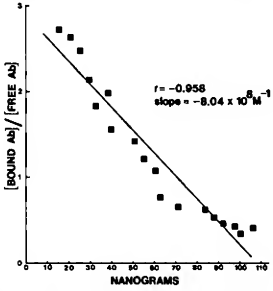

Fig. 5. Scatchard plot analysis of Lym-1 binding data with Raji cells.

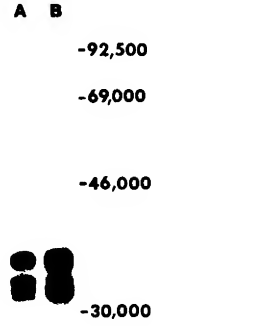

Fig. 7. Immunoprecipitation of [^{35}S]methionine- and [^{14}C]leucine-labeled Raji proteins by Lym-1 (*lane A*) and SC-2 anti-HLA-Dr (*lane B*) monoclonal antibodies.

membrane material in the nuclear preparations. Both Lym-1 and Lym-2 were such reagents and were initially identified using indirect immunofluorescence techniques using paraformaldehyde-acetone fixed cell preparations. In general, both monoclonal antibodies are reactive with circulating B lymphocytes and germinal center and mantle zone B-cells in reactive lymphoid tissues. Bone marrow and nonlymphoid tissues do not appear to be reactive with Lym-1 or Lym-2, with the exception of surface colonic epithelium which reacts weakly and variably with Lym-1 (Tables 4 and 5). Medullary dendritic cells of the thymus are also positive with Lym-1 but not with Lym-2. Both monoclonal antibodies react with surface membrane antigens which, in the case on Lym-1, has been shown by immunoprecipitation studies and competitive radioimmunoassay procedures to be related to the HLA-Dr antigen. Standard immunoprecipitation and immunoblot methods to identify the membrane antigen recognized by Lym-2 have thus far not been successful. Enzyme studies, however, have shown this antigen to be sensitive to proteases (data not shown) and further studies to identify its molecular weight are being conducted.

Lym-1 and Lym-2 are strongly reactive with the majority of B-cell derived malignancies, as demonstrated by their positive

Table 11 *Lym-1 competition studies with anti-HLA-Dr and -Ds monoclonal antibodies*

Competitive monoclonal antibody	Dilution [from stock solution (1 mg/ml)]	Bound [^{125}I]Lym-1 to 10^6 Raji cells (cpm)
Anti-HLA-Ds	None (PBS only)	3,464
	1:4	3,585
	1:8	3,101
	1:16	4,126
	1:32	2,901
	1:64	3,451
	1:128	3,397
	1:256	3,312
	1:512	3,496
	1:1,024	3,222
	1:2,048	3,946
	1:4,096	3,191
Anti-HLA-Dr	None (PBS only)	3,191
	1:4	453
	1:8	390
	1:16	531
	1:32	426
	1:64	443
	1:128	469
	1:256	1,109
	1:512	1,812
	1:1,024	2,304
	1:2,048	2,970
	1:4,096	4,061

binding to Burkitt's lymphoma, large cell lymphoma, B-cell acute lymphoblastic leukemia, and Epstein-Barr virus-transformed lymphoblastoid cell lines (Tables 1 and 2). T-cell acute lymphoblastic and myeloid leukemia cell lines as well as a wide spectrum of solid tumor cell lines (Table 3) were consistently negative by live cell radioimmunoassay procedures. Further studies using immunoperoxidase staining of frozen sections (Table 6) or immunofluorescence staining of dissociated lymphoma and leukemia biopsies using quantitative flow cytometric techniques confirmed the B-cell specificity of Lym-1 and Lym-2 (Table 7). Although Lym-1 was shown to be related to the HLA-Dr antigen, it did not bind to HLA-Dr-positive T-cell lymphomas or to activated endothelial cells and showed different, albeit similar, binding reactivities with lymphoma and leukemia cell lines when compared to standard HLA-Dr monoclonal antibody reagents. Because of these data, it is probable that Lym-1 recognizes a polymorphic variant of the HLA-Dr molecule which confers upon Lym-1 greater specificity to normal and malignant human B-cells. The binding reactivities of Lym-1 and Lym-2 shown in Tables 1 and 2 are significantly different for each other and for other published B-cell specific monoclonal antibodies, providing preliminary evidence that they recognize unique antigens expressed on human B-cells and derived malignancies. Direct comparison studies would be required to verify this point.

Radioimmunoassay methods using iodine-125-labeled Lym-1 and Lym-2 have demonstrated the presence of relatively high numbers of binding sites on target tumor cell lines. These data suggest that Lym-1 or Lym-2 conjugated to cytotoxic drugs may be effective as an alternative form of therapy since the number of binding sites per tumor cell is a critical factor in achieving significant drug concentrations at the tumor site (42). In addition, both monoclonal antibodies were shown to have high avidity binding constants in excess of 10^8 M^{-1}. Comparative studies with reactive B-cells from tonsil biopsies showed avidity binding constants with 4-fold and 2-fold lower values for Lym-1 and Lym-2, respectively, than those obtained with lymphoma target cell lines (Table 10). These data, which were obtained from measurements made independently of the binding site number, indicate that both Lym-1 and Lym-2 bind preferentially to lymphoma cells rather than to normal or reactive lymph node B-cells. The number of antibody binding sites per cell as anticipated was also higher on lymphoma cells (data not shown) which are generally larger and more homogeneous in size than reactive tonsil B-cells which range from small to large depending on their state of differentiation.

Sensitive solid-phase radioimmunoassay techniques revealed that both Lym-1 and Lym-2 recognize membrane antigens which are not shed from malignant cells grown in culture nor present free in the circulation of lymphoma patients (Table 8). Furthermore, the antigens recognized by Lym-1 and Lym-2 were not found to modulate significantly on the surface of target tumor cell lines after exposure to purified antibody preparations (Table 9).

As summarized in Table 13, Lym-1 and Lym-2 appear to have several advantages as immunodiagnostic and immunotherapeutic reagents. The data presented here suggest that these monoclonal antibodies would be useful imaging agents after conjugation with radioisotopes. As set forth by DeNardo *et al.* (43), cancer-seeking monoclonal antibodies carrying radionuclides can, in theory, be very powerful reagents for the radioimaging and radiotherapy of cancer. Optimization of this type of therapy requires dynamic modeling of physiological parameters which govern radionuclide distribution in the patient. Certain critical properties of the radiolabeled antibody must also be present in order for the target-to-nontarget uptake ratio to equate to favorable imaging and therapy. For Lym-1, the parameters for radioconjugation with radioactive iodine have been identified and experiments with this monoclonal

Table 12 *Characterization of monoclonal antibodies Lym-1 and Lym-2*

	Lym-1[a]	Lym-2[b]
Immunogen	Raji nuclei	CLL biopsy nuclei
Isotype	IgG_{2a}	IgG_1
Antigen	Protein[c]	Unknown
Antigen site	Cell surface	Cell surface
Number of antibody binding sites	1.1×10^5 sites/Raji cell	2×10^5 sites/ARH-77 cell
Antibody avidity constant	4.02×10^8 M^{-1}	2.33×10^8 M^{-1}
Lymphoid reactivity		
Lymph node and tonsil	B-cell zones and histiocytes	B-cell zones and histiocytes
Bone marrow	None	None
Blood	B-cells[d]	B-cells[d]
Thymus	Medullary dendritic cells	None
Spleen	B-cells	B-cells
Nonlymphoid reactivity	Surface colonic epithelium	None
Tumor specificity	B-cell lymphomas and leukemias	B-cell lymphomas and leukemias

[a] Studies performed with Raji Burkitt's lymphoma cells.
[b] Studies performed with ARH-77 myeloma cells.
[c] *M*, 31,000, 32,000, 34,000, and 35,000.
[d] Represents 8% of total peripheral blood mononuclear cells.

Table 13 *Advantages of Lym-1 and Lym-2 as in vivo immunodiagnostic and immunotherapeutic reagents*

Antigen
Small antigen reservoir.
Not shed nor modulated.
High number of antigenic sites per tumor cell.
Expressed on majority of human lymphomas.
Antibody
Highly stable after chemical or isotope conjugation procedures.
High avidity binding constants.
2–4-fold increase in avidity binding constants with tumor cells compared to normal B-lymphocytes.
Favorable isotype (IgG_{2a} for Lym-1) for interaction with patient immune response.

antibody indicate high retention of label and immunoreactivity after conjugation procedures (44, 45). Based upon these studies, Lym-1 has been used successfully to image lymphoma lesions in the nude mouse and in volunteer patients after iodine-123 conjugation (46). Successful radioimmunotherapy has also been achieved in lymphoma-bearing nude mice treated with single doses of [^{131}I]Lym-1 preparations (47). Because of these results, radioimmunotherapeutic trials using [^{131}I]Lym-1 have been initiated at the University of California at Davis under the guidance of Drs. Sally and Gerald DeNardo. Early results from these trials suggest that this reagent may be a powerful radioimmunotherapeutic and imaging agent. Clinical trials with unlabeled Lym-1 and Lym-2 have also been initiated at the University of Southern California to test the effectiveness and toxicity, if any, of these reagents. Finally, Lym-1 has been used to enhance NMR spin echo imaging of lymphoma-bearing nude mice after conjugation with gadolinium.[4] All of these studies emphasize the possible utility of Lym-1 and Lym-2 for the radioimmunodetection and therapy of human lymphomas. Because of their B-cell specificity, high binding avidities for human lymphomas, and stability after chemical conjugation procedures, these reagents can play an important role in delineating the necessary parameters for the successful immunodiagnosis and immunotherapy of the human malignant lymphomas after linkage with radionuclides, NMR-enhancing agents, cytotoxic drugs and toxins, or immunologically reactive peptides.

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